PLASMINOGEN ACTIVATORS IN ORAL EPITHELIUM IN VIVO AND IN VITRO

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DEDICATION

TO DR. TED. PORTER, WITHOUT WHOSE TRUST AND FRIENDSHIP NONE OF THIS WOULD HAVE BEEN POSSIBLE

C O N T E N T S

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DECLARATION

I, Yvonne Barlow, declare that the work described in this Thesis was carried out entirely by myself with the exceptions of the specialised assistance which I received in performing the electron microscopy from Mr. Robert Shields and help which I received from Dr. Nuala Booth with SDS-PAGE gels.

Signed

Date 29th November 1988

LIST OF ABBREVIATIONS

AMCHA	Amino-methyl cyclohexane carboxylic acid
ATP	Adenosine tri-phosphate
cAMP	Cyclic adenosine mono-phosphate
CPB	Citrate phosphate buffer
CSP	Cell surface protein
DFP	Di-iso-fluorophosphate
DNA	Deoxyribonucleic acid
eACA	Epsilon amino caproic acid
ECM	Extra-cellular matrix
EDTA	Ethylene-diamine-tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
Etn	Ethanolamine
FBS	Foetal bovine serum
FBSP	Foetal bovine serum proteins
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GMEM	Glasgow modification of minimum essential medium
HC	Hydrocortisone
HCG	Human chorionic gonadotrophin
Hepes	$N^1$2 Hydroxyethyl - piperazine $N^1$2 ethanesulphonic acid
^{3}HTdR	^{3}H methyl tritiated thymidine
HUVEC	Human umbilical vein endothelial cells
IBD	Inflammatory bowel disease

IL-1	Interleukin 1
KHG	Keratohyalin granules
KSCN	Potassium thiocyanate
LBS	Lysine binding sites
LH	Luteinising hormone
MAP	Microtubule associated protein
MCG	Membrane coating granules
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
NPGB	p-Nitro guanidine benzoate
PAI	Plasminogen activator inhibitor
PBSA	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Pituitary extract
PN	Protease nexin
PEtn	Phosphoethanolamine
PTFE	Polytetrafluoroethene
PTI	Pancreatic trypsin inhibitor
RA	Rheumatoid arthritis
RIA	Radio immunoassay
RSV	Rous sarcoma virus
SBTI	Soybean trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel Electrophoresis
SK	Streptokinase
TBS	Tris buffered saline
TEMED	N,N,N^1,N^1 - Tetramethylethylene diamine
tPA	Tissue type Plasminogen Activator

TGF-B	Transforming growth factor beta
UK	Urokinase
uPA	Urokinase type Plasminogen Activator

LIST OF TABLES

LIST OF FIGURES

Fig.17 Gingival biopsy. Section through basal layer and connective tissue (C). Columnar cells are interspersed with "dark cells" (x3500 mag).

Fig.18 Cytoplasm of basal cells containing ribosomes (R), rough endoplasmic reticulum (E), mitochondria (M) and few cytokeratin tonofilaments (T) (x14,000 mag).

Fig.19 Gingival biopsy. "Dark cells" within basal layer (x14,000 mag).

Fig.20 Gingival biopsy. Section through desmosomes where several cells connect in the prickle cell layer (x14,000 mag).

Fig.21 Gingival biopsy. Desmosomes between two prickle cells. Intracellular thickening can be seen forming attachment plaques into which the tonofilaments are inserted. Each desmosome shares the cytoplasm of two cells (x35,000 mag).

Fig.22 Gingival biopsy. Prickle cell layer. Increased synthetic activity in the stratum spinosum results in the production of cytokeratin tonofilaments, which group together to form tonofilament bundles (x4,200 mag).

Fig.23 Gingival biopsy. Cytoplasm of a cell in the prickle cell layer. Increased density of tonofilaments, ribosomes and mitochondria are apparent (x28,000 mag).

Fig.24 Gingival biopsy. No distinct granular layer is present, however, surface cells are characterised by dense cytoplasmic tonofilaments and a loss of subcellular organelles. Cells at surface of section contain no nuclei (x2,800 mag).

Fig.25 Vertical section through normal cultured oral epithelium after four weeks *in vitro*. Basal cells (B) attached to the surface of the dish are columnar in shape. Suprabasal cells retain their nuclei. Stained with haematoxylin and eosin (x400 mag).

Fig.26 Oral epithelial cells in culture migrating from explanted tissue (Ex) (x100 mag).

Fig.27 Confluent sheet of oral keratinocytes after 4 weeks *in vitro* (x200 mag).

Fig.28 Oral keratinocytes *in vitro*, showing spaces possibly bound by endothelial cells (x100 mag).

Fig.29 Oral keratinocytes *in vitro*. Cells migrating at the edge of an epithelial colony producing filopodia (F) and flattened lamellopodia (L) or "ruffles" (x200 mag).

Fig.30 Oral keratinocytes *in vitro*, showing dense keratinisation at the leading edge of 4 week old cultures (x100 mag).

Fig.31 Electron micrograph through cultured oral keratinocytes. Nucleated cells are present in the basal layer. Suprabasal cells are elongated, containing keratohyalin granules (kh) and surface cells contain dense cytokeratin tonofilaments (x18,000 mag).

Fig.32 Keratinocyte sloughing from the surface of the cultured cells. The detachment of keratinocytes is characterised by numerous microvilli (x18,000 mag).

Fig.33 Tangential section through oral keratinocyte *in vitro* (x2,100 mag).

Fig.34 Desmosomal junctions between cultured oral keratinocytes *in vitro* (x18,000 mag).

Fig.35 Keratohyalin granules (K)in the more superficial layer in keratinocyte cultures (x18,000 mag).

Fig.36 Cells in mitosis in keratinocyte cultures (x5,600 mag).

Fig.37 Mast cell within keratinocyte culture after 4 weeks *in vitro*. Granules display a predominantly scrolled pattern (x21,000 mag).

Fig.38 Macrophage within keratinocyte culture after 2 weeks. Macrophage is bound by keratinocytes which display prominent cytokeratin tonofilaments (x4,200 mag).

Fig.39 Keratinocytes migrating from the edge of explanted tumour tissue (ex) after 2 days *in vitro*, showing the loss of adhesion between cells (x100 mag).

Fig.40 Islands of keratinocytes growing from explanted tumour tissue after 7 days *in vitro*. Cells retain the typical cuboidal appearance of epithelial cells but fail to grow in a continuous sheet (x200 mag).

Fig.41 Almost confluent culture of oral tumour keratinocytes after two weeks *in vitro* (x400 mag).

Fig.42 Oral squamous cell keratinocytes passaged through one subculture (x200 mag).

Fig.101 Light micrograph of Fig. 100 (x100 mag).

Fig.102 Section of oral squamous cell carcinoma stained with polyclonal antiserum to human plasminogen (1:20). Fluorescence is observed in narrow cords of infiltrating tumour cells (x100 mag).

Fig.103 Light micrograph of Fig. 102 (x100 mag).

Fig.104 Section of oral squamous cell carcinoma stained with polyclonal antiserum to human plasminogen (1:20). Bright fluorescence is observed in epithelial cells within the tumour (x200 mag).

ABSTRACT

Components of the fibrinolytic system are reviewed, with particular reference to urokinase type (uPA), tissue type (tPA) plasminogen activators and plasminogen in tissues both *in vivo* and *in vitro*. Methods which may be used to demonstrate the presence of plasminogen activators in tissues are described. Both uPA and tPA were identified in histological sections of normal and malignant epithelial cells by fibrinolytic autography and immunocytochemical staining.

A method for culturing oral keratinocytes from normal gingival mucosa and from oral squamous cell carcinomas was developed. Morphological and biochemical markers of cultured cells were consistent with their tissue of origin. Cultured cells synthesized and secreted plasminogen activators predominantly of the uPA type. Plasminogen activator activity was significantly higher in gingival epithelial cultures than in tumour cultures when assayed by the fibrin plate method. However, analysis of culture supernatants after separation of plasminogen activators, from inhibitors by polyacrylamide gel electrophoresis indicated that tumour cultures contained high levels of plasminogen activator which were masked by the presence of inhibitors in the supernatants. These findings are consistent with previous observations that plasminogen activator activity is increased in malignant cells when compared with normal cells from similar tissue.

Immunocytochemical examination of normal and malignant oral epithelium demonstrated the presence of plasminogen in epithelial cells. Binding of plasminogen activators to plasminogen is known to protect plasminogen activators from the action of inhibitors and its presence in tumours may enhance local generation of plasmin by tumour plasminogen activators.

In this study, plasminogen activators have been located in normal and malignant oral epithelium. The development of an *in vitro* model, in which plasminogen activators have been identified and characterised, provides a useful tool with which to investigate the role of plasminogen activators in oral squamous cell carcinoma.

C H A P T E R 1

LITERATURE REVIEW

1.1 HISTORICAL REVIEW

1.1.1 The Discovery Of Plasminogen Activator

In the eighteenth century it was first observed that the blood of sudden death victims did not clot or if it did, then the clots dissolved spontaneously within a short period of time (Morgagni, 1769). However, it was not until very much later that the lytic activity of cadaver blood was ascribed to the presence of activators of the serum protein plasminogen.

Towards the end of the nineteenth century Denys and Marbaix (1889) made the observation that canine fibrin dissolved rapidly in serum which had been treated with chloroform indicating the presence of a lytic substance in serum. Soon after, Dastre (1893) showed that fibrin would gradually dissolve in its own serum even in the absence of an organic solvent and thus concluded that the lytic substance found was normally present in serum. He named the process fibrinolysis.

Hedin (1904) reported that the fibrinolytic activity of blood resided in the euglobulin fraction of serum and several years later Opie and Baker (1907) repeated this work and showed that the fibrinolytic activity of chloroform treated blood also resided in the euglobulin fraction of plasma.

Nolf (1904, 1905) reported that irritation of the vascular wall increased fibrinolytic activity and postulated the release of a substance from the vascular wall into the blood which enhanced fibrinolysis. Thus by the early twentieth century fibrinolytic activity was ascribed to the euglobulin fraction of serum and it was observed that this activity could be greatly enhanced after irritation of the vascular wall.

Studies of fibrinolysis, however, remained at best fragmentary until Tillet and Garner (1933) discovered that filtrates of beta haemolytic streptococci caused rapid liquefaction of human blood clots, and studies of the properties of these filtrates finally led to the identification of plasmin as the proteolytic enzyme possessing fibrinolytic activity in blood. Shortly after its discovery it was realized that the streptococcal fibrinolysin was not a true enzyme as it was unusually stable at denaturation temperatures and it did not itself digest casein or gelatin (Garner and Tillet, 1934). Later Milstone (1941) partly resolved this discrepancy when he realised that this streptococcal fibrinolysin did not lyse highly purified fibrin but required the addition of the euglobulin fraction of serum before lysis would occur and that the same euglobulin fraction would allow clots from other species to lyse. He called this accessory component "lytic factor". Christensen and Macleod (1945) further clarified matters by demonstrating that the lytic factor activated by

streptococcal fibrinolysin (later termed streptokinase) was identical to the serum protein activated by prolonged exposure to chloroform which they named plasminogen. It then became apparent that Milstone's lytic factor was in fact plasminogen, which is largely removed from highly purified fibrin.

Streptokinase was thus able to activate the precursor protein plasminogen to the active proteolytic enzyme which Christensen and Macleod named plasmin.

A paradox, however, existed in that the conversion of plasminogen to plasmin required the enzymatic cleavage of peptide bonds and yet streptokinase (SK) did not have the capacity to hydrolyse proteins. Considerable efforts were subsequently expended in resolving this issue and contributed greatly to our present understanding of the fibrinolytic system.

1.1.2 Activation Of Plasminogen - The Pro-activator Theory

Human plasminogen preparations upon treatment with streptokinase were found to give rise to two types of enzymatic activity - the ability to activate plasminogen from other species (activator activity) and plasmin activity. This observation led Mullertz and Lassen (1953) to postulate that streptokinase activation required the presence of a hypothetical proactivator which was present in human but not bovine plasminogen, which, once transformed by streptokinase, would catalytically activate plasminogen. The existence of a further lytic component was questionable since attempts to

physically separate proactivator and plasminogen consistently failed and a number of workers proposed that either plasminogen (or plasmin) and proactivator were one and the same substance (Ablondi and Hagan, 1956; Kline and Fishman, 1961; DeRenzo et al 1967).

1.1.2.1 Streptokinase-plasmin Complex

A complex between SK and plasmin, rather than plasminogen initially seemed more plausible as the proactivator since plasmin already contained an active site. Furthermore less purified preparations of plasminogen at that time always contained some preformed plasmin. By the early 1970's unequivocal physical evidence for the existence of a complex between SK-plasmin was provided by ultracentrifugal analyses (Davies, 1966; Baumgarten, 1967), column chromatography (De Renzo et al. 1967) and by discontinuous polyacrylamide gel electrophoresis (Tomar and Taylor, 1971). Kline and Fishman (1969) further elucidated the process when they found a method of differentiating between activator activity and plasmin activity in activated mixtures of SK and plasminogen, by their reaction towards pancreatic trypsin inhibitor (PTI). PTI completely inhibited the methyl lysine esterase activity of plasmin while the esterase activity of activator was unaffected. This enabled them to investigate, on a quantitative basis, the formation of plasmin or activators in reaction mixtures of SK and plasminogen. They found with the addition of large amounts of SK to plasminogen, that activator activity predominated at the

expense of plasmin. Markus and Werheiser (1964) established quantitatively the relationship between activator activity and caseinolytic activity in plasminogen preparations activated with increasing amounts of SK. They also found that because of the stochiometric interaction between plasminogen and SK, that plasmin, obtained by activation with catalytic amounts of SK, had little activator activity and high caseinolytic activity. The stochiometric complex, however, possessed full activator activity but little caseinolytic activity. Pro-activator activity was therefore identified as plasmin and activator as the SK-plasmin complex.

Detailed studies, however, on the role of spontaneously formed plasmin in the activation of plasminogen by SK showed that the activation of plasminogen did not necessarily depend on the presence of preformed plasmin. It was then suggested that another activator may exist. Independently Summaria et al (1969) and Kline and Tsao (1971) demonstrated using either diisopropyl fluorophosphate (DFP) or PTI, at levels necessary to completely inhibit preformed plasmin, that activation of plasminogen by the addition of SK still occurred. In control experiments it was shown that SK was not displacing trypsin inhibitor from the plasmin inhibitor complex, thus the observed activation could not be due to the participation of plasmin.

1.1.2.2 Streptokinase-plasminogen Complex

The hypothesis that the interation of human plasminogen with SK results in the formation of a SK-plasminogen complex, with enzyme activity, was attractive. However, to establish

this, it had to be demonstrated that a complex between SK and plasminogen existed and that such a complex had the enzymatic properties of an activator.

Reddy and Markus (1972) re-examined the role of spontaneous plasmin in reaction mixtures using the burst reagent p-nitro guanidine benzoate, NPGB, (Chase and Shaw, 1970) which reacts as an active centre site specific compound yielding nitrophenol and the inactive acylated enzyme. If SK is mixed with plasminogen in the presence of excess NPGB activation of the sample still occurs. They also showed a linear correlation existed between the amount of plasminogen remaining for activation and the amount of SK added; ie. if SK and plasminogen were added in an equimolar ratio no activatable plasminogen remained after removal of NPGB. Further evidence for the enzymatic properties of the SK-plasminogen complex were provided by Reddy and Markus (1974) and later by Wohl et al (1977) who reported that the complex could hydrolyse appropriate synthetic substrates.

Based on their studies, Markus and Reddy described the mechanism of activation of plasminogen (Fig. 1) as follows:

(i) SK reacts with plasminogen to form an equimolar complex which acquires the properties of an enzyme by exposing an active centre in the plasminogen moiety without cleavage of peptide bonds.

(ii) The enzymatic SK-plasminogen complex then generated catalyses the conversion of

FIG. 1

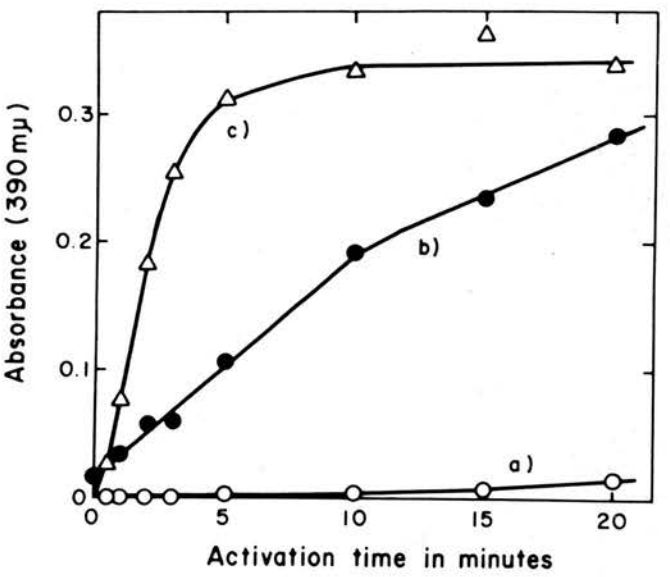
Effect of NPGB - inhibition of the spontaneous plasma content of plasminogen on activation rates by streptokinase.

(a) Spontaneous plasmin inhibited : activation with low levels of streptokinase.

(b) Spontaneous plasmin not inhibited : activation with low levels of streptokinase.

(c) Spontaneous plasmin inhibited : activation with high levels of streptokinase.

From Reddy and Markus, 1972


Absorbance (390mμ)
0.3
0.2
0.1
0
c)
b)
a)
0
5
10
15
20
Activation time in minutes

plasminogen to plasmin by splitting the arginyl-valyl bond in the plasminogen molecule.

(iii) Cleavage of this same bond in the SK-plasminogen complex converts it to SK-plasmin.

Thus if catalytic amounts of SK are used to activate plasminogen the products will be plasmin and an amount of SK-plasmin equivalent to the amount of SK used for the activation. If, however, an equimolar amount of SK is added to plasminogen then only modified SK-plasmin will be formed.

To date no other activators isolated from vertebrates function in this manner. Thus, whilst the discovery and use of streptokinase was historically very important in expanding the field of fibrinolysis it also probably served to confuse the issue of plasminogen activation in higher animals by vertebrate activators.

1.2 THE BIOCHEMISTRY OF PLASMINOGEN

Central to the understanding of plasminogen activation has been the determination of the amino acid sequences of the region of the plasminogen molecule in which proteolytic cleavage generates plasmin. The inactive zymogen, plasminogen, is present in blood plasma at a concentration of approximately 3uM (Robbins and Summaria, 1970; Collen et al 1972; Linjen and Collen, 1982) and is maintained at a relatively constant level with a half life of approximately

2.2 days. The isolation of native plasminogen from plasma has long been complicated by its marked tendency to stick to other proteins (Remmert and Cohen, 1949; Kline, 1953) and by its sensitivity for partial proteolytic degradation by traces of plasmin generated during purification (Wallen, 1962). An important step in the elucidation of the biochemistry of plasminogen was the development of an effective purification procedure, using affinity chromatography, in which lysine coupled to Sepharose was used as an adsorbent of plasminogen (Deutsch and Mertz, 1970). Several modifications of this technique have subsequently been used and current methods of working at near neutral pH in the presence of the low molecular weight inhibitor aprotinin have yielded essentially pure preparations of highly stable plasminogen (Wallen and Wiman, 1970, 1972). In the last ten years the complete amino acid sequence of the plasminogen molecule has been determined (Wallen, 1977; Wiman and Wallen, 1975; Sottrup-Jensen et al 1978a, b). Native plasminogen consists of a polypeptide chain composed of 790 amino acid residues, M_r 92000, and has glutamic acid as a single NH_2 terminal amino acid and asparagine as the COOH terminal residue, the whole molecule being stabilised by 24 disulphide bridges (Fig.2). Plasminogen also contains approximately 2-3% carbohydrate which is located in the A chain of the molecule and this may contribute to the microheterogeneity found in even highly purified preparations of plasminogen.

Plasminogen, containing glutamic acid as the N terminal acid, was found to be electrophoretically identical to

FIG. 2

The primary structure of glu-plasminogen.

Red = A (heavy) chain.

Yellow = B (light) chain.

Orange = N-terminal peptides released on generation of Lys-plasminogen.

from Wiman, 1978

plasminogen from fresh plasma and has been termed native or Glu-plasminogen (Summaria, 1976). Glu-plasminogen is readily converted to another form by partial digestion with plasmin (Claeys et al 1973) which is the result of cleavage of one of three bonds in the N terminal region - Met 68, Lys 77 or Arg 78 - leading to the appearance of a new N terminal acid (Fig.2). These forms, which are collectively termed Lys-plasminogen, have an M_r of about 8000 daltons lower than native plasminogen and differ markedly in their functional properties. Proteolytic release of N terminal peptides causes gross conformational changes in the Lys-plasminogen molecule with activation of Lys-plasminogen occurring at a considerably faster rate than Glu-plasminogen (Christensen and Mullertz, 1977). Activation of plasminogen to the active protease plasmin occurs as the result of cleavage of a single peptide bond Arg_{560} - Val_{561} in the plasminogen molecule. The conformational change brought about in the Lys-plasminogen molecule appears to make this bond more easily accessible. In vivo activation of plasminogen can be brought about by a number of activators and although the mechanism of action may be different, the resulting structural changes in the plasminogen molecule are very similar (Summaria, 1967). Two questions arise concerning the enzymes involved and the sequence of events. Firstly, whether enzymatic release of N terminal peptides and cleavage of the Arg_{560} - Val_{561} bond are the result of activator activity or whether the cleavage of the NH_2 terminal region of the molecule is effected

autocatalytically by plasmin. Secondly, whether Glu-plasminogen is converted to Lys-plasminogen first or whether the two cleavages occur at random. Analysis of the plasminogen derivatives occuring on activation of plasminogen have shown that in addition to Glu-plasminogen, Lys-plasminogen and Lys-plasmin were also present (Wiman and Wallen, 1973). In experimental conditions designed to minimise autocatalytic digestion by plasmin, no Glu-plasmin was found in the reaction products and based on these experiments Wiman and Wallen (1973) suggested that activation of plasminogen occurs in two consecutive steps:

$$\text{Glu-plasminogen} \xrightarrow{1.} \text{Lys-plasminogen} \xrightarrow{2.} \text{Lys-plasmin.}$$

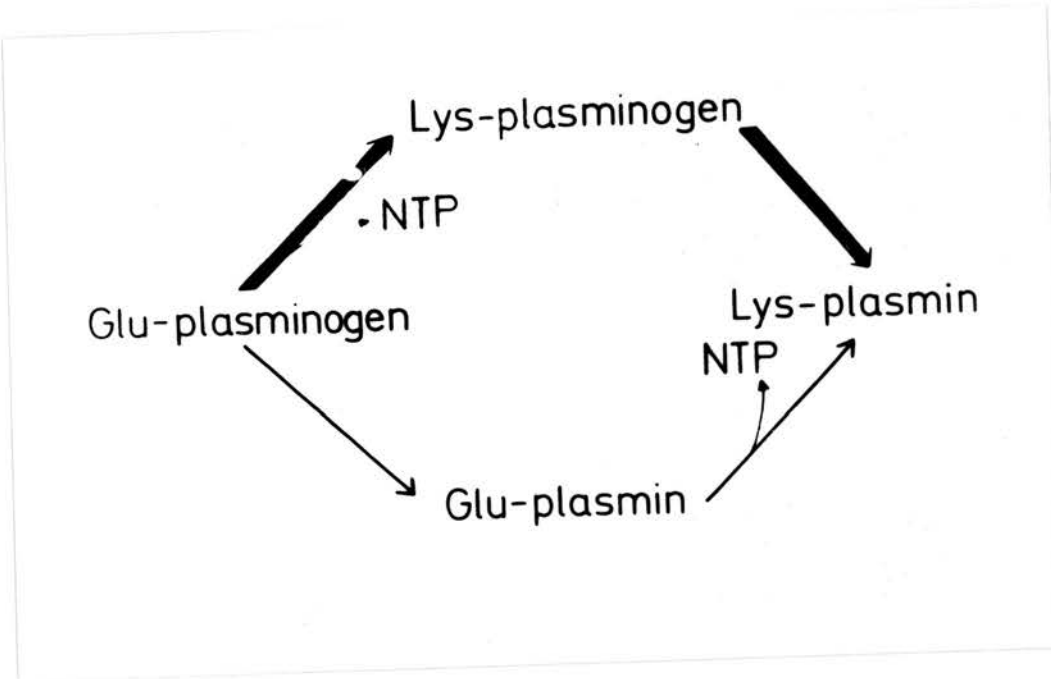
Later, however, Sodetz et al (1974) found that by activating plasminogen in the presence of the plasmin inhibitor aprotinin, no peptides were released from the N-terminal end of the molecule and therefore the activator did not seem to cleave bonds in the heavy chain of the Glu-plasminogen molecule. The two pathways of activation were subsequently suggested by Violand and Castellino (1976) and both proceed in two steps as outlined in Fig. 3.

Glu and Lys-plasminogen are converted to Glu or Lys plasmin respectively by cleavage of the single Arg_{560} - Val_{561} bond by plasminogen activator. Small amounts of plasmin, which are present during the initial stages of activation (in the absence of inhibitors), then catalyse the conversion of Glu-plasminogen to Lys-plasminogen and of Glu-

FIG. 3

Pathways of activation of glu-plasminogen.

Heavy arrows indicate possible major pathway.


Lys-plasminogen
NTP
Glu-plasminogen
Lys-plasmin
NTP
Glu-plasmin

plasmin to Lys-plasmin. As previously noted, the activation of Lys-plasminogen occurs at a much faster rate, the formation of Lys-plasminogen acting as a feedback mechanism that amplifies plasminogen activation. In experimental systems cleavage of N terminal peptides from Glu-plasminogen precedes activator catalysed conversion of Lys-plasminogen to Lys-plasmin but it is not clear yet whether Glu-plasmin or Lys-plasmin is the physiological thrombolytic enzyme.

1.3 PLASMIN

The active protease plasmin consists of two polypeptide chains held together by disulphide bonds (Fig. 4). The light chain with an M_r of approximately 25,000 contains the active site and has amino acid sequence homologies with other serine proteases such as trypsin and elastase. Plasmin has broad specificity hydrolysing proteins and peptides at lysyl and arginyl bonds (Castellino and Powell, 1981; Robbins et al 1981). Plasmin formation is essentially an irreversible process because in common with other hydrolytic reactions proteolysis is an exergonic reaction and under normal physiological conditions there are no simple mechanisms to repair a broken bond. This type of enzyme formation is also more rapid than that regulated by selective transcription of the genome and is triggered by signals operating entirely at a post translational level. Formation of the highly aggressive plasmin enzyme must therefore be controlled at all stages (Fig.5). Control of formation of plasmin can occur

FIG. 4

Arrangements of chains in different types of plasminogen and plasmin.

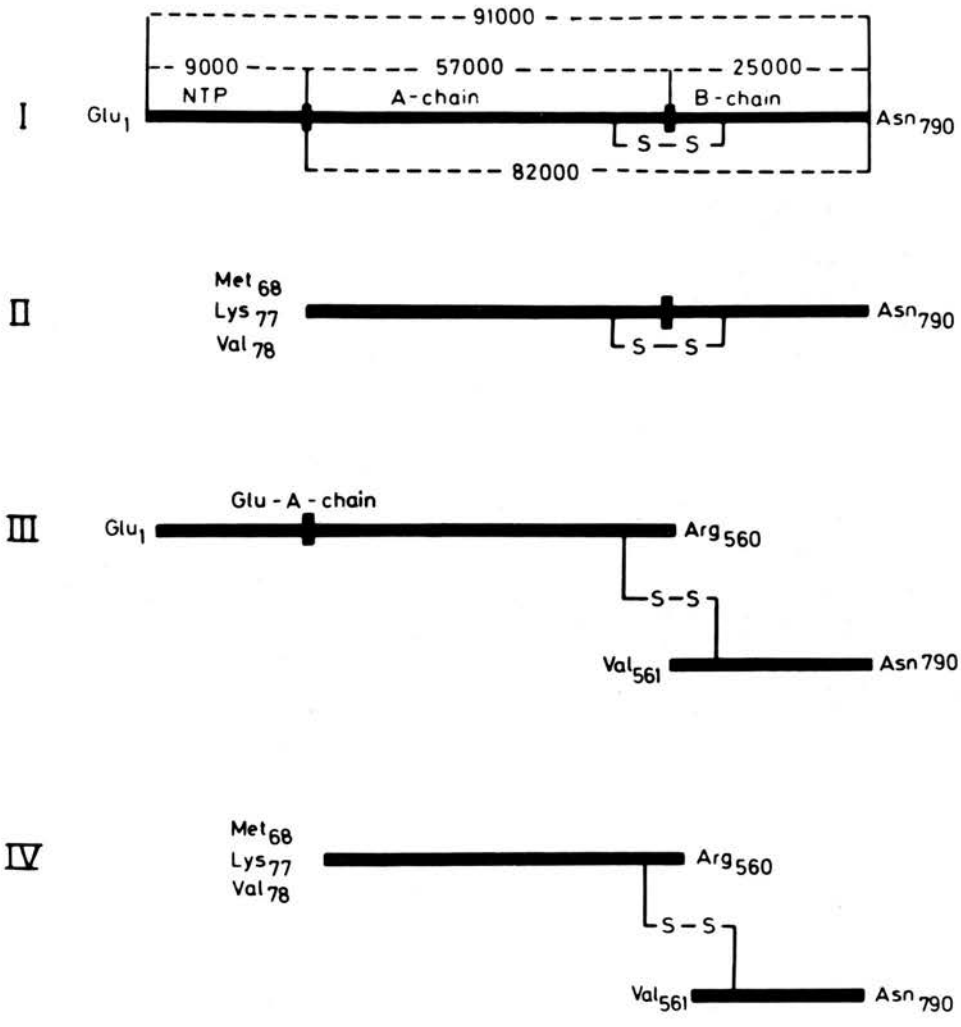
I Glu-plasminogen

II Lys-plasminogen

III Glu-plasmin

IV Lys-plasmin

From Kline and Reddy, 1983


I
91000
9000
57000
25000
NTP
A-chain
B-chain
Glu1
Asn790
S — S
82000
II
Met 68
Lys 77
Val 78
Asn790
S — S
III
Glu - A - chain
Glu1
Arg560
S-S
Val561
Asn 790
IV
Met68
Lys77
Val 78
Arg560
S — S
Val561
Asn 790

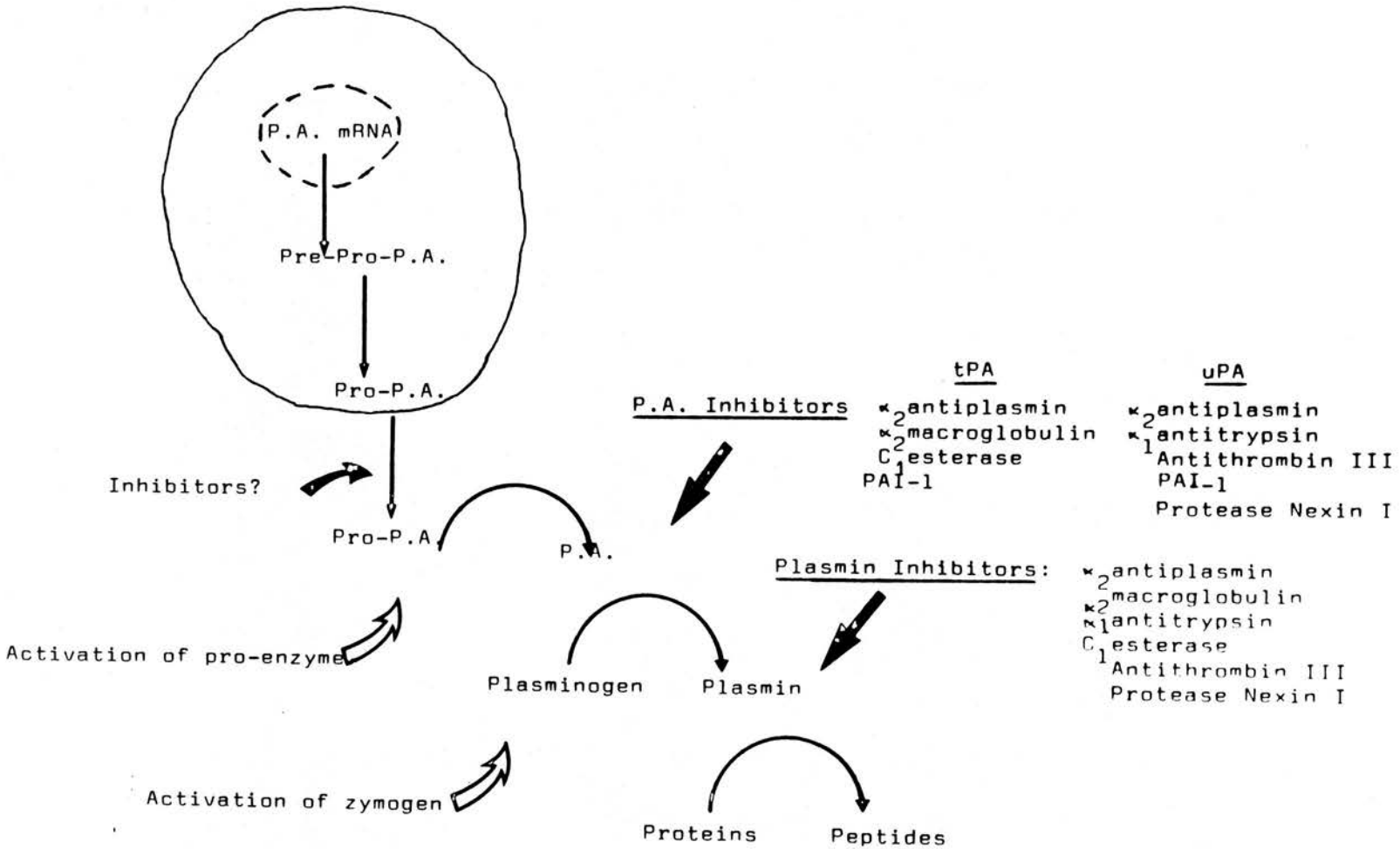


FIG. 5

Regulation of plasminogen activation.

either in the cascade reaction before the formation of plasmin, or by inhibitors of plasmin, once it has been formed (Collen et al 1983).

There are five well defined plasma proteins which operate as plasmin inhibitors; alpha-2-antiplasmin, alpha-2-macroglobulin, alpha-1-anti-trypsin, anti-thrombin III and C1 esterase. The two quantitatively most important inhibitors are alpha-2-antiplasmin and alpha-2-macroglobulin. Alpha-2-antiplasmin is an extremely fast acting inhibitor of plasmin and alpha-2-macroglobulin is thought to be of importance only when local depletion of alpha-2-antiplasmin occurs (Travis and Salvesen, 1983). In the presence of normal concentrations of these two inhibitors, the other plasma protease inhibitors do not play a role in the inactivation of plasmin (Mullertz, 1974), however, they are known to participate in the inhibition of plasminogen activators. Recently a cellular inhibitor of plasmin, protease nexin I (PN I), secreted by a number of cell types has been detected (Knauer and Cunningham, 1984; Farrell and Cunningham, 1986). These inhibitors appear to act at the pericellular level but are functionally related to circulating anti-thrombin III (Scott and Baker, 1983). Because plasmin is so rapidly inactivated it has been proposed that for fibrin degradation to occur that plasminogen must be bound to fibrin (Aoki et al 1983). Plasminogen activation would therefore only occur on the fibrin surface where the plasmin would be protected from inactivation by plasma inhibitors. Several studies have confirmed that plasminogen does indeed bind to fibrin and a

number of investigators have established that interaction with fibrin occurs at the lysine binding sites in the plasminogen molecule (Thorsen et al 1981; Lucas et al 1983). Furthermore, plasminogen activators have also been found to adsorb to clotted blood which would further localise the generation of plasmin and confine its activity. Indeed, plasminogen activators have been found not only to bind to the fibrin surface but to markedly enhance the incorporation of plasminogen, particularly Lys-plasminogen, into the fibrin clot (Linjen et al 1981; Harpel et al 1985).

1.4 METABOLISM OF PLASMINOGEN

A decisive factor in the local formation of plasmin is the presence of the inactive zymogen, plasminogen. Approximately 40% of total plasminogen in mammals is located extravascularly and has been demonstrated in a number of locations including eosinophils (Barnhart and Riddle, 1963), granulocytes (Prokopowicz and Niewiarowski, 1968), bovine follicular fluid (Beers et al 1975), ascites fluid in mice (LeBlanc and Black, 1975), human and porcine uterine fluid (Casslen and Ohlsson, 1981), human saliva (Moody, 1982) and epidermis (Isserof and Rifkin, 1983; Nakagawa et al 1984). However, details of the sites of synthesis and storage of plasminogen are still unclear.

Isotopic labelling and organ perfusion techniques have shown that the liver is the primary site of synthesis of the

majority of plasma proteins and perturbation of levels of plasminogen in patients with liver disease indicated a function for the liver in the synthesis of plasminogen (Fletcher et al 1964; Davis and Picoff, 1969). The early work of Barnhart and Riddle (1963) showed that eosinophils from bone marrow contain plasminogen and subsequently Prokopowicz and Niewiarowski (1968) found that it was contained in all granulocytes, where it has similar properties to plasma plasminogen. Data presented later by Prokopowicz and Stormorken (1968) indicated that biosynthesis of plasminogen occurred within the granulocytes although their ability to release stored plasminogen was not confirmed. However, the observation that plasminogen levels in guinea pig granulocytes decreased rapidly after treatment with protease inhibitors suggests that this may be likely.

Highsmith and Kline (1971) reported that the kidney is a major site of synthesis of plasminogen in plasminogen depleted cats. After a 10 hour delay, presumably for the synthesis of plasminogen, normal levels were restored within 18 hours from depletion. These authors also found a large negative arterial-venous difference in plasminogen concentration across the kidney during the restoration phase following acute depletion, which further supports the contention that the kidney has a major role in plasminogen biosynthesis. Furthermore, bilateral nephrectomy of their animals blocked the return to normal plasminogen levels which consistently occurred after depletion of intact animals. The authors considered it unlikely that the release of

plasminogen from granulocytes could account for this large increase in circulating plasminogen but they suggested a regulatory function for granulocytes in normal turnover. Although bilateral nephrectomy prevented the return of normal plasminogen levels in depleted cats, Siefring and Castellino (1975) reported that synthesis of plasminogen occurred in the absence of kidneys in the rat and further showed, by incorporation of labelled proteins, that synthesis was de novo. In another study using a rat liver perfusion system Saito et al (1980) demonstrated the cumulative appearance of plasminogen in the perfusate. Evidence for de novo synthesis in the liver was provided by incorporation of ^{14}C labelled leucine into specific immunoprecipitates of plasminogen and by inhibition of this by cycloheximide.

Evidence indicating that the liver was also the primary site of synthesis of circulating plasminogen in man was provided by Raum et al (1980). They determined genetic types of plasminogen in a liver donor and recipient before hepatic homotransplantation. In plasma samples obtained from the recipient after transplantation it was shown by isoelectric focusing in polyacrylamide gels that the plasminogen type had been changed to that of the donor.

Whilst the liver may therefore be the primary site of circulating plasminogen in man, granulocytes have also been shown to contain, if not synthesize, plasminogen (Prokopowicz and Stormorken 1968). More recently plasminogen has been located in epidermis (Isseroff and Rifkin 1983; Nakagawa et

al 1984) and in testes (Saksela, 1986). A function for plasminogen at these sites remains to be elucidated.

1.5 PLASMINOGEN ACTIVATORS

Plasminogen activators are widely distributed in nature occurring in microorganisms (most notably streptococci and staphylococci species) and throughout the vertebrates, although their distribution appears to exhibit some species specificity.

The two activators of major importance found in humans are tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA).

1.5.1 The Biochemistry Of Plasminogen Activators

1.5.1.1 Urokinase Type Plasminogen Activator

The discovery of large amounts of uPA in urine made it an attractive source for purification. Simple affinity procedures coupled with the use of monoclonal antibodies to uPA have led to the purification of this enzyme to apparent homogeneity (Kaltoft et al 1982; Vetterlein and Calton, 1983).

Human uPA occurs in a one chain and two chain form, the latter being the active form of the enzyme. Active uPA has an M_r of approximately 50,000 and is composed of two subunits, M_r 20,000 and M_r 30,000, linked by one disulphide bond (Sumi and Robbins, 1983). Studies of the amino acid

sequence have shown that the active site is located in the M_r 30,000 peptide. High and low molecular weight forms of the active enzyme exist both of which are identical with respect to the heavy B chain. The difference in molecular weight appears to be due to limited proteolytic cleavage occuring in the M_r 20,000 chain (Gunzler et al 1982a) although the functional significance of this is not known.

1.5.1.2 Tissue Type Plasminogen Activator

As tissue PA constitutes such a small proportion of total protein, multi-step purification procedures have led to very low yields and only partially purified preparations (Kok and Astrup, 1969; Rickli and Zaugg, 1970; Aoki, 1974). In this respect, cultured cell lines, particularly melanomas, have been a valuable source of tPA and it would appear that Bowes melanoma activator is identical with that found in human uterine tissue (Rijken and Collen, 1982; Wallen et al 1983). Native tPA is a single polypeptide with an M_r of approximately 70,000 (Nielsen et al 1983; Pennica et al 1983) and unlike uPA sometimes occurs in an active form. Digestion with plasmin yields a two chain form with disulphide bonding in the A and B chains. The B chain contains the active site which is homologous with the active chains of other serine proteases. Human tPA occurs in two variants of slightly different relative mass (Wallen et al 1983; Andersean et al 1984) due to differences in glycosylation of the A chain of the molecule.

1.5.1.3 Genes Coding For uPA And tPA

The complete amino acid sequence of uPA has been

determined by Gunzler et al (1982b), and its comparison with the amino acid sequence of tPA led them to conclude that the two activators were products of different genes. This has since been confirmed by the work of Rajput et al (1985) who, using cDNA probes, were able to map human tissue PA to chromosome 8 and uPA to chromosome 10. This, however, disagrees with the earlier work of Kucherlapati et al (1978) who located tPA to chromosome 6. Kucherplati and his colleagues relied on the expression of the PA gene as a means of determining its presence, while Rajput et al used hybridisation of structural gene sequences to specific cDNA probes, thus avoiding the uncertainty that a regulatory gene was being identified in hybrids. The different distribution of these two enzymes in tissues further supports the contention that these enzymes have different functions.

1.5.2 Tissue Distribution Of Plasminogen Activators

Tissue PA is mainly located in tissues and has been detected in endothelial cells, gastric mucosa, lung, pituitary (Kristensen et al 1984, 1985) thyroid, kidney, uterus, prostate (Rijken et al 1981) and brain (Soreq and Miskin, 1981; 1983). Tissue PA secreted from endothelial cells into the blood is the major thrombolytic enzyme (Wun and Capuano, 1985). Consistent with its role in the removal of fibrin from arteries and veins, tPA has also been located in fluids in other channels eg. uterine fluid (Granelli-Piperno and Reich, 1978), tears (Thorig, 1983),

seminal plasma (Rijken et al 1981), human milk (Okamoto et al 1981) and bile (Oshiba and Ariga, 1983). Urokinase type PA, detected first in urine because of its relatively high concentration in this fluid, has since been found, albeit in lower concentrations, in blood plasma (Shakespeare and Wolf, 1979), semen (Rijken et al 1981), uterine fluid (Casselsen et al 1981) and bile (Oshiba and Ariga, 1983). Immunocytochemical studies have shown the tissue distribution of uPA to differ from tPA, being widely distributed in connective tissue and in epithelial cells lining organs and ducts; particularly kidney, bladder, prostate and involuting mammary glands (Larsson et al 1984; Camioli et al 1984; Nakamura et al 1984).

Generally serine proteases are released as inactive pro-enzymes (Neurath and Walsh, 1976) but it was not until Skriver et al (1982) obtained a preparation of one chain uPA from conditioned medium of 3T3 cells that latent uPA was definitively demonstrated. Pro-uPA was unable to activate plasminogen or to incorporate DFP. Since then other laboratories have detected pro-uPA (Nielsen et al 1982; Eaton et al 1984) and recently it was reported that freshly removed urine contained 50% of its uPA in the inactive form (Kielberg et al 1985).

The finding that uPA can be released in an inactive form demonstrates the existence of another step in the cascade reaction leading to the formation of active plasmin, although the regulatory mechanisms which act at this stage are unknown (Fig.5).

1.5.3 Plasminogen Activators In Fibrinolysis

Some studies have suggested the existence of cellular non-plasminogen substrates for uPA. Quigley (1979) has demonstrated alterations in cellular behaviour which occur as a result of plasminogen activators but independently of plasmin, although the site of action of plasminogen activator is unkown. Keski-Oja and Vaheri (1982) have isolated from cells a protein (M_r 66,000), which acts as a substrate for plasminogen activator although its cellular location remains unkown. Apart from these two studies, the only well documented protein substrate for both uPA and tPA is plasminogen although the enzymes do differ in their affinity for fibrin. Plasminogen and thrombin both bind to fibrin with binding sites located in characteristic triple disulphide loops or kringle structures (Patthy et al 1984). The A chain of tPA contains two kringle structures which partly accounts for the fibrin binding properties of tPA (Pennica et al 1983). In addition the 43 amino acid residue located in the terminal portion of the A chain has regions homologous with the finger domains responsible for the fibrin binding properties of fibronectin (Banyai et al 1983). Although uPA has no similar finger domain counterpart in its molecule (Gunzler et al 1982b) it does contain one kringle structure in the N terminal part of the single chain form. Pro-uPA was found to have a much greater binding affinity than uPA and was efficient at clot lysis at concentrations that did not result in removal of systemic fibrinogen (Kasai

et al 1985). Pro-uPA is thus a more specific and better thrombolytic enzyme than uPA, but its contribution in vivo is not clear. The greater fibrin binding affinity of tPA makes this the likely thrombolytic enzyme in vivo and the fact that tPA, secreted by the endothelial cells lining the blood vessels, is increased after venous occlusion (Nilsson and Robertson, 1968), exercise or systemic administration of agents such as adrenalin (Cash, 1975; 1978), further supports this hypothesis. The rapidity with which the increase in plasminogen activator is observed, approximately 10 minutes, suggests a release of tPA rather than an increase in the synthesis of tPA. It is possible, however, that there is activation of a pro-enzyme form. Indeed abnormalities in tPA levels in the blood have been implicated in a number of vascular disorders (Stead et al 1983; Booth et al 1983; Booth and Bennett, 1984; Nilsson et al 1985). Tissue PA is also the better thrombolytic enzyme in experimental systems, and clinical trials using tPA have shown this therapy to be valuable in patients with coronary thrombosis without concomitant alterations in systemic fibrinogen or alpha-2 anti-plasmin. The dangerous bleeding tendency caused by the systemic lytic effects with other thrombolytic agents such as streptokinase can therefore be avoided (Matsuo et al 1981; Korninger et al 1982; Van der Werf et al 1984). As a result tPA is now being produced in several laboratories using recombinant DNA technology (Pennica et al 1983; Edlund et al 1983; Brown et al 1985).

Tissue PA mediated activation of plasminogen is known as the extrinsic or cellular pathway, however, blood also contains an intrinsic or humoral plasminogen activation pathway to generate plasmin. Evidence has recently been reported for pathways activated independently by Factor XII, complement, kininogen and platelets (Kaplan and Yecies, 1980; Kline and Reddy, 1980), all of which may subsequently enhance plasminogen generation by the extrinsic pathway. These activators of plasminogen appear in plasma when clotting Factor XII (Hageman Factor) is activated (Ratnoff and Rosenblum, 1958). Activation of Hageman Factor involves proteolytic cleavage of the native molecule into one or more fragments which activates pre-kallikrein to kallikrein, which in turn activates plasminogen to plasmin. Kallikrein also releases vasoactive peptides from kininogen which participate by enhancing the rate of activation of Hageman Factor. Although plasmin and kallikrein activate Hageman Factor, it is not known how this occurs because they exist as precursors until Hageman Factor itself is activated. The existence of pathways other than Hageman Factor dependent pathways have been suggested by a number of studies. Plasma deficient in kallikrein activates plasminogen, although at a slower rate, and Goldsmith et al (1977) have suggested that Hageman Factor itself can activate plasminogen directly. Astrup and Rosa (1974) also found that plasma, deficient in Hageman Factor, exhibited normal levels of fibrinolytic activity when dextran sulphate was added to plasma. Schreiber and Austin (1974) have also observed that C3 deficient plasma

did not sustain clot lysis. Plasma deficient in C2 allowed normal clotting indictating that the reaction is not dependent on classical C3 convertase, as did plasma deficient in C6 indicating that the terminal C5-C9 complex was not required. Their results suggested that C3 was a factor in the lysis of clots but the mechanism was unknown. Earlier Taylor (1973) observed that thrombin and Factor Xa modify the platelet membrane at the time of polymerisation of fibrinogen. Recruitment of the complement component C3 onto the membrane is followed by platelet fusion and clot retraction, indicating one possible role for complement in thrombolysis. It would appear that multiple pathways exist for the generation of plasmin in blood so that the absence of a factor need not have serious consequences for normal clotting.

1.5.4 Other Functions Of Plasminogen Activators

The very wide distribution of plasminogen activators in tissues suggests that there may be biological processes in which activators participate other than fibrinolysis and a number of studies have provided evidence for their involvement in processes requiring extracellular proteolysis.

A prerequisite for this function is their ability to degrade components of the extracellular matrix. In addition to proteoglycans, the extracellualr matrix contains several well defined proteins; laminin, fibronectin, elastin and several types of collagen. Plasmin is able to degrade

fibronectin and laminin. Collagenous components within the matrix, themselves resistant to plasmin digestion, may be hydrolysed by latent collagenases which can be activated by plasmin (Paranjpe et al 1980; O'Grady et al 1981). It has also been reported that in vitro degradation of elastin and collagen in the extracellular matrix by elastase and collagenase is facilitated by plasmin mediated removal of other glycoproteins (Jones and DeClerk, 1980; Chapman et al 1984).

The presence of a large and widely distributed extracellular reservoir of plasminogen makes the hypothesis that plasminogen activators are involved in tissue degradation feasible. Correlation with respect to time and hormonal regulation between plasminogen activator production and normal physiological events requiring limited proteolysis corroborate this hypothesis. Interactions between cells and components of the extracellular matrix are essential for tissue differentiation and regulation during embryogenesis, for maintenance of normal tissue differentiation and in tissue remodelling (Beers et al 1975; Vassalli et al 1976; Strickland et al 1976; Ossowski et al 1979; Valinsky et al 1981).

At the time of ovulation, a close relationship has been observed between the disruption of the follicle wall, releasing the ovum, and plasminogen activator activity (Beers, 1975; Strickland et al 1976; Shimada et al 1983). Secretion of plasminogen activators by granulosa cells occurs only hours before ovulation. Tissue PA, but not uPA, is

stimulated by the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH), which are also elevated in the blood prior to ovulation. Ny et al (1985) have found that an inhibitor of plasminogen activator is also produced by granulosa cells. However, the same gonadotrophins which stimulate tPA also suppress this anti-activator activity. Gonadotrophin induction of tPA and suppression of anti-activator may therefore be sufficient for the formation of plasmin, and hence activation of collagenases (Reich et al 1985), leading to the disruption of the follicle wall. The testicular counterpart of granulosa cells, the Sertoli cells, are also believed to secrete plasminogen activators under hormonal control. These are involved in the release of primary spermatocytes from the basal lamina, and the liberation of mature spermatids into the adluminal compartment (Lacroix et al 1979).

Rapid degeneration of several tissue components also occurs during mammary gland involution after lactation. In murine species this involution correlates with the production of activators by mammary epithelial cells (Ossowski et al 1979). Recently, direct evidence for uPA in mammary epithelial cells has been demonstrated immunocytochemically by Larsson et al (1984). Furthermore, hormones which counter post-lactational involution, also inhibit uPA production. Controlled tissue degradation occurs during attachment of the embryo to the uterine wall. In mouse, trophoblasts secrete plasminogen activators during the

invasive growth phase (Strickland et al 1976). During the early embryonic phase tPA is secreted by the parietal endoderm and uPA by the visceral endoderm and mesoderm (Marotti et al 1982). In the later stages tPA is synthesized by endodermal cells but does not appear to have any obvious function in tissue degradation (Strickland and Madhavi, 1978). For clarification of the roles of tPA and uPA in any of these processes in vivo, selective inhibition of tPA or uPA enzyme activity would contribute greatly to our understanding of their function.

Secretion of activators has also been implicated in the migration of cells during embryogenesis, particularly haemopoetic cells into the Bursa of Fabricus (Valinsky et al 1981) and endothelial cells in response to angiogenic factors (Gross et al 1983a). More recently Moonen et al (1982; 1985) observed increased plasminogen activator activity in the developing nervous system and this activity is thought to be involved in neuronal migration. Both uPA and tPA are produced in the developing cerebellum although uPA alone is able to induce astrocyte proliferation in cultured explants of nervous tissue. Production of both plasminogen activators was decreased in adult material and the authors suggested a developmental regulation in the expression of genes for plasminogen activators. Cyclical fluctuations in plasminogen activator activity also occur during the differentiation of normal keratinocytes in culture (Isseroff et al 1983), however, their role in maturation and shedding of

keratinocytes has not been established with certainty.

1.6 PLASMINOGEN ACTIVATORS IN NON-NEOPLASTIC CONDITIONS

The abnormal formation of plasmin has been implicated in a number of diseases, particularly vascular disorders, dermatological conditions and diseases involving inflammatory processes where mechanisms regulating activators produced by these tissues are not controlled.

1.6.1 Vascular Disorders

An important factor in the pathogenesis of venous thrombosis is a defective fibrinolytic system (Sundqvist et al 1981) due to a decrease in vessel wall activator or defective release of the activator. A recent study of patients with recurrent idiopathic deep vein thrombosis found that the predisposition to thrombosis was, in some patients, the result not only of reduced tPA levels but also because of a concomitant increase in inhibitor to PA ratio (Nilsson et al 1985). At the other extreme, the only well characterized bleeding disorder is due to a deficiency of the principal plasmin inhibitor, alpha-2-anti-plasmin (Aoki et al 1979; Kluft et al 1979). However, Booth et al (1983) have described a new haemorrhagic disorder due to excessive production of tPA, where no abnormality of platelet function or coagulation was demonstrable. It would appear then that faults in the regulation of plasminogen activators alone can result in vascular disease. Abnormal plasminogen activator activity

has long been associated with some of the secondary effects of diabetes mellitus such as arteriosclerosis and retinopathy. In the literature conflicting evidence is cited of increased (Lassman et al 1974), normal (Cash and McGill, 1969) and decreased (Almer and Pandolfi, 1976) fibrinolytic activity in diabetics. Ishibashi et al (1985) suggest that studies of localized fibrinolytic activity may be more important than generalized fibrinolysis in the development of diabetic retinopathy. They reported that decreased plasminogen activator activity in the retinae of diabetic rats leads to a reduction in the ability to control microthrombus formation, resulting in occlusion of the retinal vascular bed.

1.6.2 Dermatological Conditions

Farb et al (1978) presented evidence of an enzymatic mechanism which was active in the induction of acantholysis in cultured keratinocytes by pemphigus IgG. They found that cell detachment induced by pemphigus IgG could be blocked by protease inhibitors (SBTI and alpha-2-macroglobulin) as did Singer et al (1980) and they suggested that the loss of cell adhesion was due to proteolytic activity. Schlitz et al (1978) has previously demonstrated that this enzyme activity was not of lysosomal origin. Later Hashimoto et al (1982, 1983) found elevated plasminogen activator activity in keratinocytes incubated with both pemphigus vulgaris and pemphigus foliaceous IgG. Furthermore they demonstrated, using Todd's technique of fibrinolytic autography, that

cutaneous fibrinolytic activity could be inhibited by e-amino caproic acid (eACA), which further implicated plasminogen activator. Later Morioka et al (1987) found that acantholysis induced in skin explants by their incubation with pemphigus vulgaris and pemphigus foliaceous IgG, could be blocked by the addition of anti-urokinase antibody. The molecular mechanisms leading to the release of plasminogen activator are unknown.

Increased proteolytic activity in epithelial cells has also been reported in rapidly proliferating psoriatic epidermis (Fraki et al 1982) and most probably results from an increase in tPA which has been found in abundance in shed psoriatic scales (Grondhal-Hansen et al 1985). Although the mechanism resulting in activation of plasminogen is unkown, psoriatic epidermis maintains its increase in specific activity of plasminogen activators even after grafting epidermis onto nude mice (Fraki et al 1982).

Corneal ulceration after alkali burns to the eye is the result of a fault in regulation of plasmin produced at the corneal surface to effect resorption of fibrin after injury (Wang et al 1982). Increased levels of plasminogen activators cause a persistent epithelial defect to develop leading eventually to ulceration. The activators are produced by epithelial cells but also by polymorphs which infiltrate the cornea after injury and amplify the plasminogen activator dependent destruction of tissue. The role of plasminogen activators in inflammation has been documented in a number of

diseases.

1.6.3 Inflammation

Vassali and his colleagues (1976; 1979) first discovered that cultured stimulated macrophages or peritoneal macrophages harvested from sterile inflammatory exudates produce and release uPA. This process can be inhibited by glucocorticoids and other anti-inflammatory steroids. There is some evidence to suggest that PA facilitates migration of inflammatory cells, including polymorphs, which also secrete uPA, to the site of inflammation (Reich, 1978). This function of macrophages in other inflammatory conditions has since been noted.

Patients with chronic inflammatory bowel disease (IBD) ie. Crohn's disease and ulcerative colitis, have lesions characterized by an infiltrate in which macrophages are prominent. The secretion of plasminogen activators by cultured macrophages from patients with active IBD is significantly greater than in age matched subjects without the disease. This activity is also significantly greater in untreated patients compared to treated patients (Doe and Dorsman, 1982). In protein losing enteropathy, in which the the intestinal mucosa is also ulcerated, increased tPA secretion in the gastric mucosa appeared to enhance mucosal permeability. It was found that drugs inhibiting plasmin, such as amino-methyl cyclohexane carboxylic acid (AMCHA), were effective at blocking the membrane disorder although the mechanism of increased production of tPA was not clarified

(Kondo et al 1976).

Toki et al (1982) reported increased fibrinolytic activity as a result of uPA synthesis in the skin lesion of allergic vasculitis and suggested that uPA might originate from the polymorphs which infiltrated the lesion and perhaps from blood released into the tissue space after disintegration of the vascular wall.

Excessive production of plasminogen activator by mononuclear cells and polymorphs has been found in the rare condition Chediak-Higashi syndrome, leading to profound coagulation abnormalities. This appears to be the result of increased numbers of cells producing activators rather than an increase in synthesis and secretion of activators (deSaint Basile et al 1985).

Although evidence is circumstantial in many instances, loss of control of plasmin production has been implicated in a number of diseases in which inflammatory processes occur.

Of the inflammatory diseases, rheumatoid arthritis (RA) is a condition which afflicts a large percentage of the population. Plasminogen activator has been detected in human synovial fluids and membranes, and in cultures of enzyme dissociated rheumatoid tissues (Werb et al 1977). Berger in 1977 postulated that a defect in the secretion of plasminogen activator from RA synovial tissue was the cause of perpetuation of the inflammatory process. Hamilton (1982) found, however, that early passage synovial fibroblast

cultures from RA and non-RA patients both had low levels of plasminogen activator activity. It was suggested that even if basal secretions between the two populations had differed, that the observation may not be relevant *in vivo*. Extracellular plasmin from synovial cells would be quickly complexed and might be unavailable for activity. Such plasmin-alpha-2-antiplasmin complexes have already been detected in the joint spaces of RA patients (Clemmenson *et al* 1977). They suggest that a significant degree of plasminogen activator activity may result from a modulation of synovial cell activity *in vivo*. Indeed, in both RA and non-RA fibroblast cell lines, plasminogen activator activity can be stimulated by conditioned medium from peripheral blood mononuclear cells (Hamilton and Slywka, 1981; Hamilton, 1982). This may be one example of an *in vivo* regulator of plasminogen activator activity.

The rheumatoid synovial pannus is not only inflammatory but is hyperplastic and invasive (Zvaifler, 1977). The fact that the pannus erodes and replaces cartilage in much the same fashion as tumour destroys normal tissue has meant that "aggressive" and "tumour-like" properties have been ascribed to it (Linder, 1975). Macrophage mediated elevation of synoviocyte PA levels, in the RA pannus, possibly by interleukin 1 (Mochan *et al* 1986), may contribute to the tissue remodelling and cell migration. Hamilton (1983) further proposed that the active moiety from stimulated macrophages may mimic to some extent the effects of tumour promoters on cell function. Indeed, elevation of PA is often

an early event associated with cellular transformation.

1.7 PLASMINOGEN ACTIVATORS IN NEOPLASIA

Proteolytic degradation of the extracellular matrix and the basal lamina by neoplastic cells has been considered for a number of years to be involved in the destruction of surrounding tissues and the invasiveness of tumours. Accumulating evidence would suggest that in general proteolytic activity in tumour tissue is higher than in its normal counterpart, but, reports to this effect are by no means unequivocal (Dano, *et al* 1985).

1.7.1 Plasminogen Activator In Neoplastic Tissue

The fibrinolytic activity of tumour tissue was documented during the early twentieth century (Carrel and Burrows, 1911). Fischer (1925) later correlated the rapidity of clot lysis with the neoplastic state of tumour tissue but failed to demonstrate the enzymatic nature of the phenomenon. The use of the fibrin overlay technique (Todd, 1959) confirmed Fischer's observations, plasminogen activator activity being detected in a number of malignant tissues; invasive squamous cell carcinoma (Weiss and Beller, 1969), ovarian tumours (Svanberg *et al* 1975), human colon tumours (Newstead *et al* 1976) and even in dysplastic cervical cells (Jensen *et al* 1979). No distinction was made between uPA and tPA at this time, and since fibrin stimulates tPA activity, it is possible that mainly tPA activity was being detected

using this technique. Enzymatic analysis of tumour extracts have also indicated the presence of plasminogen activator of an undetermined type in human kidney and bladder carcinomas (Ladehoff, 1962) bronchial carcinomas, prostatic, cervical and ovarian tumours (Nagy et al 1977; Chylak and Brdar, 1983) and breast cancers (Peterson et al 1973) but since tPA is found in endothelial cells lining vessels, it is likely to be found in most tissues and the significance of these findings should not be over-estimated. More specifically, tPA has been found in murine tumours (Mira-y-Lopez et al 1983) and in extracts of human colon tissues (Tissot et al , 1983) although the same reservations apply.

Urokinase-type plasminogen activator has been detected in lung, colon and breast carcinomas, in tumour aspirates and in virally induced tumours (Corasanti et al 1980; Markus et al 1980; Niklasson et al 1981; Evers et al 1982; Mira-y-Lopez et al 1983).

1.7.2 Plasminogen Activator In Neoplastic Cell Cultures

Much of the interest in the role of plasminogen activators in tumour invasion and metastases resulted from the finding that large numbers of cultures of neoplastic origin have been reported to release elevated levels of plasminogen activator (Dano, et al 1985), with far fewer reports of cultures of neoplastic origin containing amounts of plasminogen activator similar to those found in normal tissues or lacking any detectable activator (Wilson and

Dowdle, 1978; Reckliesetal1980; Gronow and Blenheim, 1983). More recently reports have established that some cell lines produce uPA (Dano et al 1980; Wilson et al 1980, 1983; Strickland et al 1983) and other lines tPA (Rifkin et al 1974; Rifken and Collen, 1981; Ornstein et al 1983; Strickland et al 1983). Infrequently neoplastic cultures have been reported to produce both types of activator although it is unclear whether both activators are produced in the same cells or whether each activator is synthesized by a different cell population (Wilson et al 1980, 1983; Strickland et al 1983; Schleuning and Reich, 1983).

Furthermore the neoplastic transformation of many cultured cells coincides with a marked increase in extracellular proteolytic activity due mainly to the release of plasminogen activators although other serine proteases and collagenases have also been detected (Chen and Buchanan, 1975; Dano et al 1980).

1.7.3 Transformation Of Cell Cultures

Malignant transformation (Schaeffer, 1979) describes the transition in vitro of non neoplastic cells into neoplastic cells capable of forming tumours. Such transformation can be induced either by viruses or chemical carcinogens (Heidelberger, 1973; Graham et al, 1974; Barret et al 1978) or in some species it may occur as a result of spontaneous events (Sandford and Hoemann, 1967). The relationship between transformation in vitro and malignancy in vivo is not clear. Normal cells, which can be transformed in vitro, sometimes

fail to produce tumours when inoculated back into the host. Similarly, cultured cells derived from malignant tissues can themselves undergo transformation resulting in further alterations in growth characteristics. Since malignancy itself cannot be demonstrated *in vitro*, we are obliged to use the properties expressed by cultured cells derived from tumours, or cells made tumorigenic, as indicators of malignancy. Transformation results in a series of biochemical alterations, which can include increased proteolytic activity (Dano *et al* 1985) enhanced agglutination (Burger, 1969; Inbar and Sachs, 1969) increased sugar uptake (Hatanaka and Hanafusa, 1970), a reduction in serum concentration required for growth (Holley and Kiernan, 1968; Lindgren, 1975) and a decrease in cyclic nucleotides (Goldberg *et al* 1975; DeLarco and Todaro, 1978). Changes in the underlying biochemistry of the transformed cell result in a morphological phenotype which is associated with transformation (Pastan and Willingham, 1978) and is characterized by cells rounded in shape, an increase in cell surface irregularities and disordered alignment of cells. Most of these phenomena are correlatedly expressed and are unlikely to be under phenotypic control. These changes are largely due to diminished cell adhesion. The loss of cell to substrate adhesion is reflected in the ability of many malignant or transformed cells to grow in suspension or semi solid media. Furthermore, loss of adhesion leads to reduced cell-cell recognition resulting ultimately in loss of density limitation (contact inhibition) and a disordered

growth pattern (Otten et al 1971; Anderson et al 1973; Carchman et al 1974). Two molecules which may be important in controlling the transformed phenotype are cyclic AMP (cAMP) and cell surface protein (CSP) (Hynes, 1976; Keski-Oja et al 1977). Many normal lines show an increase in cAMP at confluency which transformed lines often fail to do when crowding occurs. Treatment of many transformed cell lines with cAMP results in cell flattening and elongation, decreased agglutination and decreased surface irregularities (Willingham et al 1973). Cyclic AMP is thought to act by phosphorylation of key proteins. Two phosphoproteins, filamin and microtubule asssociated protein (MAP), are responsible for maintaining cell shape and are located at the plasma membrane (Willingham and Pastan, 1974; Davies et al 1977). These proteins may therefore be susceptible to the action of cAMP. The assembly of microfilaments is increased by cAMP and by CSP, both of which enhance adhesion, and it seems likely that disruption of these structures in transformed cells is the result of decreased adhesion rather than a defect in microfilament assembly. The secretion of large amounts of cell surface protein by normal cells is often reduced in their transformed counterparts and although CSP increases cell adhesion (Olden&Yamada1977; Adams et al 1977) it has no metabolic effects on growth, hexose transport or saturation density (Yamada et al 1976; Yamada and Pastan, 1976). The increase in proteolytic activity which often accompanies transformation may assist in removing CSP from the surface of cells.

The role of proteases in transformation is generally less clear. Enzymes and substrates located at the surface of the cell on the plasma membrane mediate a variety of cellular interactions including cell adhesion, cell recognition and cell-cell communication and factors affecting growth control. Trypsin, exogenously added to cultures can stimulate density limited fibroblasts to undergo another round of cell division (Vaheri et al 1974; Blumberg and Robbins, 1975). This surface protease effect on cellular mitosis has been demonstrated in normal and neoplastic tissues (Bosmann, 1972) and several laboratories have indicated that the addition of proteases to cultures can cause the cells to mimic some of the properties of transformed cells. Further evidence that proteases might be responsible for some of the characteristics of transformed cells results from experiments in which the addition of protease inhibitors to the growth medium of cultures of transformed cells helped to restore properties associated with normal cells (Borek et al 1979; DiPaolo et al 1980; Markus, 1984).

In genetic studies using Rous sarcoma virus (RSV), rapid morphological and metabolic changes occur in cell behaviour when the transforming gene src is expressed (Bader, 1972; Anderson et al 1973; Yoshida et al 1977). This gene has the capacity to code for a protein 40-60,000 daltons but it seems unlikely that the one gene product could be responsible for all the properties of transformed cells and an alteration of some basic control mechanism has been suggested. At an early

stage in transformation the src gene product affects the integrity of the cell surface, possibly mediated by proteolysis of membrane proteins or by cAMP. Recent evidence has suggested that the src gene product may be a polypeptide hormone (Brugge and Erikson, 1977; Jay et al 1978) which initiates changes resembling those which occur in cultures on addition of growth factors appropriate to that cell type (glucose transport is also increased). Addition of polypeptide hormones to cell cultures has been known for some time to stimulate synthesis of plasminogen activators (Lee and Weinstein, 1978) thereby producing a factor which has already been noted under certain circumstances to disturb normal metabolism.

Much work is required to elucidate the nature of the changes occurring on transformation of cells. Evidence would seem to indicate, that the increase in proteolytic activity associated with transformed cells, not only has the potential to enhance tumour invasion, but can induce alterations in growth control and cell metabolism.

1.7.4 Plasminogen Activator Production In Transformed Cultures

The relationship between transformation by oncogenic viruses and plasminogen activator synthesis has been thoroughly studied by infection with Rous sarcoma virus (RSV). Unkeless et al (1973) showed that chick embryo fibroblasts consistently developed extracellular proteolytic activity after transformation by RSV. Proteolytic activity

was not present in normal cells or in cells after infection with non transforming strains of the virus. In cells infected with temperature sensitive mutants proteolytic activity only appeared after the cultures were incubated at permissive temperatures. The proteolytic activity was later shown to be caused by induction of plasminogen activator which activated plasminogen in the medium (Quigley et al 1974; Unkeless et al 1974; Rifkin et al 1975). This activator was evaluated by its M_r to be uPA (Granelli-Piperno and Reich, 1978).

Viral transformation may alter the host cell regulation of plasminogen activator synthesis. Ossowski et al (1973) using a strain of RSV that transformed both chick and rat embryonic fibroblasts found an increase in uPA in both cell types but each type of uPA showed species specificity for plasminogen. Urokinase-type plasminogen activator must therefore be determined by the host cell.

Balduzzi and Murphy (1975) found, using wild type, mutant and non transforming RSV, that only viruses capable of causing morphological transformation induced plasminogen activator synthesis. Wolf and Goldberg (1976), however, found no difference in the degree of morphological changes in clones producing low, intermediate or high levels of plasminogen activator although no evidence was produced in these studies to indicate the presence of pro-enzymes or inhibitors. Since O'Donnel-Tormey and Quigley (1981) found a protease inhibitor in RSV transformed fibroblasts, which would inhibit release of plasminogen activator from cells it

is not surprising that conflicting results are sometimes reported.

In general transformation by DNA oncogenic viruses, causing extracellular proteolysis, is less consistent than that obtained by RNA viruses particularly RSV (Dano et al 1985). Selection procedures for these transformed clones are very elaborate and cultivation periods are often extensive. Because of selection which occurs during culture procedures particularly from tumours which have such a heterogenous population of cells and because repeated cloning also accentuates phenotypic and genotypic differences, studies based on the use of cell cultures must always be interpreted with caution.

1.7.5 The Role Of Plasminogen Activators In Neoplastic Tissues

Transformed cell cultures and those derived from malignant tissues generally secrete higher levels of plasminogen activator than normal cells. Tumour tissue *in vivo* also exhibits high extracellular proteolytic activity (Dano *et al* 1985). This property of tumour cells may promote tumour invasion and metastases. To firmly establish a causal role for plasminogen activator in tissue degradation by tumours, plasminogen activator activity of tumour tissue need not necessarily be higher than in normal tissues but should ideally be found where invasion into surrounding tissue is taking place. Secondly protease inhibitors should also inhibit metastases and reduce tumorigenicity of

malignant cells. Studies using inhibitors specific to each type of activator should help to clarify the role, if any, of each type of activator in tumour biology.

1.7.6 Location Of Plasminogen Activators In Tumour Tissues

The recent development of specific antibodies to tPA and uPA has allowed localization of activators in tissues and should give a clearer indication of the role of these enzymes in tumour invasion. In the limited number of studies so far performed using these antibodies, uPA immunoreactivity has been demonstrated in colon tumours and melanomas (Khoga et al 1985; Burtin et al 1985; Markus et al 1983, 1984), prostatic cancer (Camiolo et al 1984) and murine lung tumours (Skriver et al 1984). In the latter study and that of Khoga et al (1985) the number of cells containing uPA were greatest in areas where tissue degradation was occurring and is therefore consistent with its putative role in tumour invasion. Extractable uPA from colon (Markus et al 1983) and murine tissues (Skriver et al 1984) was not significantly higher than in normal tissues. However, in normal colon fewer cells stained positively with uPA antibodies than in tumour tissue. Such discrepancies may be resolved by better control of possible cross reactivity with other antigens in tissue sections and by better quantitation of the pro-enzyme or inhibitor levels. Many more studies such as these are required before a direct relationship between plasminogen activator and extracellular proteolysis in tumours can be

established.

1.7.7 Inhibition Of Tumour Metastases

In several experimental systems leupeptin and antipain, both plasminogen activator inhibitors, reduce carcinogen and radiation induced transformation of cells (Borek et al 1979; DiPaolo et al 1980; Kennedy and Little, 1980; Kinsella and Radman, 1980). Leupeptin has also been found to reduce lung metastases in the rat after injection of hepatoma cells (Meyn et al 1977). It should also, however, be noted that some studies have failed to show this inhibition. The role of inhibitors has been extensively reviewed by Markus (1984).

Specific inhibition in relation to metastases has been attempted in only one study. Ossowski and Reich (1983) found that antibodies which specifically inhibited the enzyme activity of human uPA but not chicken uPA decreased metastases of human tumour (Hep-3) to chicken lung after transplantation onto the chorioallantoic membrane of chick embryos. Since growth at the site of inoculation of the tumour was not inhibited, then uPA potentially plays an important role at an early stage in the metastatic process. Another approach to the role of plasminogen activators in the development of tumours is to compare the production of plasminogen activators by cell lines for their metastasizing capacity and their tumorigenicity. Wang et al (1980) found that subline F10 of the B16 melanoma line which secretes more plasminogen activator than subline F1 had greater frequency of lung metastases after subcutaneous inoculation although

other laboratories have been unable to detect differences in metastatic capacity using the same cell lines (Nicholson et al 1978). Similarly a study of the metastatic capacity of clones of Lewis lung tumours was found in one case to be positively correlated with high levels of plasminogen activator (Eisenbach et al 1985) while in another study no correlation was found (Whur et al 1980). Similar conflicting evidence surrounds the tumorigenicity of certain transformed cell lines (Jones et al 1975a, b; Gallimore et al 1977) when injected back into either nude mice or immunoincompetent animals. Several laboratories have found good correlation between the secretion of plasminogen activator and the ability to form tumours (Pollack et al 1974, 1975; Laug et al 1975; Ossowski and Reich, 1980; Adelman et al 1981; Bruggen et al 1981) while other studies have indicated the correlation to be less pronounced or absent (Jones et al 1975a; Becker et al 1977; Gallimore et al 1977; Wolf and Goldberg, 1978; Tenchini et al 1983). Conflicting results in these kinds of studies are not surprising since no account can be taken of local environmental factors especially inhibitors and hormones, which can regulate cellular synthesis of plasminogen activator. The hormonal milieu of the tumour may be especially important since many tumours are capable of synthesizing their own hormones (Todaro and DeLarco, 1976), hence their reduced serum requirement in vitro.

1.8 REGULATION OF PLASMINOGEN ACTIVATOR ACTIVITY

Plasminogen activator activity is regulated by the rate of biosynthesis of the pro-enzyme, the release of pro-enzyme from the producer cells and the conversion of the pro-enzyme to plasminogen activator. At each of these stages the presence of stimulatory or inhibitory activity will affect the final level of plasminogen activator activity detected in a given tissue.

1.8.1 Conversion Of Pro-enzyme

Mechanisms which regulate the activation of pro-enzymes have previously been considered unimportant because of the presence of trace amounts of plasmin in extracellular spaces likely to be responsible for the conversion of released pro-enzyme. However, Skriver et al (1984) found, in Lewis lung tumour, that all of the extracellular uPA was in the one chain form. In addition Kielberg et al (1985) later demonstrated that at least 50% of the uPA in the urine drawn from the murine bladder was present in the inactive form. Mechanisms as yet unknown must therefore exist which control the conversion of the pro-enzyme to the active form.

1.8.2 Synthesis Of Plasminogen Activator

Glucocorticoids suppress uPA and tPA activity in many tissues although suppression of tPA tends to be much more variable. The possible role of inhibitors in steroid induced

changes in uPA may be investigated by separation of these components using gel electrophoresis. Involvement of inhibitors, which either bind covalently or migrate at the same position on the gels cannot be ruled out but results obtained suggest that changes in the concentration of inhibitors are not the only reason for steroid effects. Steroids appear to regulate plasminogen activator levels by regulating the rate of gene transcription. RNA synthesis inhibitors block induction of plasminogen activator hence there is a requirement for de novo synthesis of mRNA. RNA synthesis inhibitors also inhibit glucocorticoid suppression of intracellular levels of uPA and tPA probably by inducing an intracellular regulatory molecule which in turn inhibits plasminogen activator gene transcription or mRNA translation (Rifkin and Crowe, 1980).

In many cell types, plasminogen activator activity is regulated by polypeptide hormones, epidermal growth factor (Lee and Weinstein, 1978; Eaton and Baker, 1983), luteinising hormone, follicle stimulating hormone and human chorionic gonadotrophin (Strickland and Beers, 1976), prolactin (Miray-Lopez et al 1983) and calcitonin (Sims et al 1981; Sudol, 1985). Given that cAMP is the likely intracellular second messenger for these hormones, other compounds known to increase cAMP for example cholera toxin (Rheinwald and Green, 1977) are found to enhance or mimic the effects of these hormones. These compounds also effect the synthesis of plasminogen activator in a number of cell cultures many of which show parallel changes in plasminogen activator levels

with cAMP and hormones (Dayer et al 1981; Soreq et al 1983). In a similar manner to steroid hormones, cycloheximide and actinomycin D block cAMP and growth factor induction of plaminogen activator by a mechanism dependent on mRNA synthesis.

1.8.3 Inhibition Of Plasminogen Activator Activity

Both uPA and tPA are inhibited by the circulating plasma protease inhibitors (Fig. 5), however, the significance of these inhibitors in neutralization of activators in the blood is unknown because reaction rates between inhibitors and activators are much lower than the clearance rates in vivo would suggest (Waller et al 1983).

Until recently the presence of specific inhibitors of plasminogen activators have never been convincingly demonstrated because of the lack of appropriate kinetic data. However, the existence of plasminogen activator inhibitors has now been accepted (Sprengers et al 1986) and these have been characterised into three immunologically distinct groups; PAI-1 (endothelial cell type inhibitor), PAI-2 (placental inhibitor) and protease nexins. PAI-1 has been demonstrated in plasma (Kruithof et al 1983) and in culture fluids of normal and transformed cells lines (Van Mourik et al 1984; Sprengers et al 1985; Kruithof et al 1984; Schleef et al 1985), although its precise function in the regulation of fibrinolytic activity is still only partly understood. The

inhibitors in some cases have been tested only with uPA or tPA but some reports indicate the inhibition of both activators (Erikson et al 1984; Lecander et al 1984; Van Mourik et al 1984). Korninger and Collen (1981) have reported second order rate constants for the reaction between activator and these inhibitors in plasma. These are several orders of magnitude higher than the rates of reaction between plasminogen activator and the abundant plasma protease inhibitors (Ranby et al 1982) and high enough to allow the reaction rate within the plasma to be higher than the observed clearance rates in vivo. The tissue distribution of these inhibitors is unknown.

Recently it has been demonstrated that co-cultivation of cell types can result in the reduction or disappearance of plasminogen activator from culture media. Co-cultivation of mouse melanoma cells secreting tPA with a non secreting sub clone of the same cell type leads to the disappearance of tPA from the culture medium (Kyner et al 1978). Similarly co-cultivation of a rat neuroblastoma stem cell line with its tumorigenic plasminogen activator secreting descendent also results in the reduction in the synthesis of plasminogen activator (Liu et al 1984). In both cases no inhibitor was involved in this process. Hoal et al (1983) found that the disappearance of tPA from melanoma cell lines co-cultivated with normal fibroblasts was the result of attachment of the tPA to a surface component of fibroblasts. Knauer and Cunningham (1984) have reported at least three molecules termed protease nexins (PN I, PN II, PN III) which are

secreted by fibroblasts into culture medium where they form covalent linkages with proteases. PN I is known to complex with uPA (Baker *et al* 1980) and with plasmin (Eaton and Baker, 1983). Protease nexins synthesized *in vivo* would therefore be able to remove certain proteases from their extracellular environment and PN I in particular may function to regulate endogenous uPA or plasmin. These complexes readily bind to the cells which secrete them and are rapidly internalised and degraded (Knauer and Cunningham, 1984). More recently Saksela *et al* (1985) reported similar cell derived factors produced by a fibrosarcoma cell line. They found not only the secretion of a heparin binding protease similar to protease nexin but also another unidentified inhibitor of uPA.

Such interactions between various cell types which produce a change in metabolism of plasminogen activator are of obvious importance in connection with their function in neoplasia. Enhancement or inhibition of local proteolytic activity by contact between various cell types may be involved in determining whether a tumour may metastasize and in which place secondary tumours are likely to arise. Such mechanisms also suggest a possible alternative to plasminogen activator production by malignant cells themselves.

1.9 SUMMARY

Evidence is accumulating that plasminogen activators and particularly uPA may be involved in pericellular proteolysis

during tumour invasion and metastasis. Elevated levels of plasminogen activator are associated with tumour tissue *in vivo*, and increased plasminogen activator activity is displayed by many cultures derived from malignant tissues or by normal cultures after transformation. Control of protease production in the cascade reaction is complex. The rate of synthesis of activators can be controlled at the transcriptional or translational level. After pro-activators are secreted from cells, activation to the two chain form is influenced by regulatory mechanisms, as yet unknown, or by inhibitors of plasminogen activators. The production of the protease plasmin is similarly under inhibitor control. Plasminogen activator synthesis is extremely sensitive to physiological concentrations of hormones and growth factors. Events which increase the rate of synthesis of plasminogen activators or shift the inhibitor balance in tumour areas are largely still unknown. These events presumably depend to some extent on tumour type. In the last few years attempts have been made to identify uPA or tPA in certain tissues and tumours and the recent development of monoclonal antibodies to uPA and tPA should allow laboratories to separately locate these molecules in tissues.

Furthermore, recognition of the importance of attempting to measure pro-enzyme and inhibitor levels in tissue should help elucidate events which result in the final concentrations of plasminogen activators in different tissues under various conditions.

1.10 OBJECTIVES

In this study the role of plasminogen activators and plasminogen in oral squamous cell carcinoma was investigated. The objectives were as follows:

1. To develop appropriate techniques for the culture of gingival epithelium and epithelium from the squamous cell carcinomas.

2. To study the presence of plasminogen activators and their inhibitors in oral tissues *in vivo* and *in vitro*.

3. To determine levels of plasminogen activators in cultures of normal and malignant oral epithelium.

4. To study the presence of plasminogen in oral epithelium and oral squamous cell carcinomas.

CHAPTER 2

TISSUE CULTURE

2.1 INTRODUCTION

Tissue culture evolved as a natural progression from early embryological work. Traditional culture procedures in use today were established in the first two decades by workers such as Carrel and Burrows (1911) and subsequently a great variety of techniques became available as investigators modified these methods for their own experiments. The full potential of tissue culture was gradually realised and by the 1950's the culture of animal cells was employed in such diverse areas as virus replication, morphogenetic and transplantation experiments, the study of neoplasia and more recently in drug testing, cytogenetic analyses and hybridoma technology.

The successful growth of cells in culture depends on the provision of suitable environmental and nutritional conditions (Paul, 1975; Waymouth *et al*, 1981; Freshney, 1987). By far the most important single factor in culturing cells is the growth medium, whose function is to provide the physical conditions of pH and osmotic pressure and the complex chemicals required for growth. The first major contribution in this area was made by Eagle (1955) who defined the basic nutritional requirements of cells in culture. This was followed by the formulation of many different synthetic media

optimised for different cell types. The basic constituents and functions of media for animal cells are outlined below.

2.1.1 Media

2.1.1.1 Carbohydrate

Glucose is included in most media as the carbon source of choice. It is metabolised principally by glycolysis to produce lactic acid and via the Krebs cycle to form carbon dioxide. Galactose may be substituted and has the advantage that its metabolites are less likely to cause acidification of the medium, which is particularly evident with transformed cell lines. Sugars can also be replaced by simpler molecules, for example lactate or pyruvate, which will satisfy the energy requirements of the cells given an adequate oxygen supply.

2.1.1.2 Salts and Buffers

Most animal cells require sodium, potassium, calcium, magnesium, chloride, phosphate and bicarbonate ions for growth. Sodium and potassium are mainly required for the maintenance of osmotic pressure, whilst calcium and magnesium are necessary for certain intracellular enzymes to function and appear to promote attachment of cells to their substrate. Low calcium levels have been found to favour proliferation of epithelium and inhibit fibroblast growth in epithelial cell cultures (Peehl and Ham, 1980a,b; Tsao *et al*, 1982; Boyce and Ham, 1983).

Of the cations, phosphate is essential for the production of ATP by the cells, and bicarbonate, which functions in a number of fundamental biochemical processes, is the main buffering system in culture. Medium buffered with bicarbonate does, however, require equilibration with 5% carbon dioxide in the gas phase. The use of other buffers is complicated by the fact that the cells have a metabolic requirement for carbon dioxide and these buffers may sometimes be used in conjunction with lower concentrations of bicarbonate. The overall concentration of inorganic ions in the medium is constrained by the total osmotic pressure in which the cells can survive. In practice human cells in vitro can tolerate osmolalities between 260-320 mOsm kg^{-1} (human plasma is ca.290 mOsm kg^{-1}) (Freshney 1987).

2.1.1.3 Amino Acids

The ten essential amino acids in the mammalian diet are included in all media plus (usually) cysteine, tyrosine and glutamine. Non essential amino acids commonly used in the synthesis of proteins are available commercially and can be added to media, particularly if cells are cultured at low clonal density.

2.1.1.4 Vitamins and Organic Compounds

Minimal media contain the B group of vitamins, choline and i-inositol,the other requirements generally being met by serum. Polyunsaturated fats, also required by most cells, are derived from lipids bound to the serum proteins.

2.1.1.5 Serum

The importance to the medium of serum as a contributor of factors, many of which are as yet undefined, is underlined by the requirement of cells for additional hormones, vitamins, growth factors, non essential amino acids, and organic compounds, such as linoleic acid and polyamines, in serum free media.

Serum is composed mainly of binding proteins which recognise vitamins, lipids and hormones and which may stabilise and modulate the action of the substances which they bind.

Serum contains a variety of growth factors, albeit in low concentrations, essential to cells; epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and mitogen stimulating activity (MSA). Growth factors are mitogenic and some are thought to exert their effect by binding to insulin receptors on cell surfaces thus promoting the uptake of glucose. Hydrocortisone, a steroid hormone also found in serum, is known to promote cell attachment, cell proliferation and to induce differentiation. The concentrations of these and other factors may vary from one serum batch to another and the effect of serum batch on cell growth has been widely recognised (Barnes and Sato, 1980).

Other serum proteins sequester toxic substances (Iscove and Melcher, 1978) and the purity of the water becomes very important in serum free media.

Serum also provides protease inhibitors which protect cells from damage during the passage procedure, and substitutes for this function, for example soybean trypsin inhibitor, must be provided when serum is omitted.

Lastly, serum modifies the surface of the substrate to which cells attach by depositing a layer of fibronectin (Hook et al 1977) and many cell lines require the addition of a fibronectin coating if cultured in the absence of serum.

The requirement of virtually all primary and established cell lines for serum has hampered investigation of many aspects of cell biology. Cells growing in a defined medium would be of major importance since they represent the ideal system for the study of drug and hormone action, growth and differentiation in the absence of undefined factors.

2.1.2 Substrate

Most cells, excluding some haemopoetic and transformed cell lines, require to spread out on a substrate in order to proliferate. A critical parameter for such anchorage dependence is the density of negative charges on the surface of the substrate. In this respect glass has been largely superceded by sterile disposable plastics, the surfaces of which are charged by etching with sulphuric acid. Attachment and growth of many cell types is improved by the application of a number of surface coating materials: PTFE, cellulose nitrate, collagen, fibronectin, laminin and extracellular matrix (Lui and Karasek, 1978; Savage and Bonney, 1978; Gospadarowicz et al 1980; Hawley-Nelson et al 1980; Lillie et

al 1980; Clark et al 1985; Lenoir et al 1985). Some of these materials not only improve growth but may promote differentiation and influence the expression of tissue specific functions.

2.2 CULTURE OF EPITHELIAL CELLS

Most tissue culture systems were originally designed for fibroblasts which can be routinely cultivated through many generations as unchanged diploid cells (Hayflick and Moorhead, 1961) or indefinitely as transformed cell lines.

Two major problems are associated with the cultivation of keratinocytes; one is the overgrowth in primary culture of keratinocytes by the more vigorous stromal elements in mixed tissues, and the other more exacting problem is the inability to continuously subculture epithelial cells.

Many approaches to the problem have included seeding epithelial cells on growth arrested feeder layers, the provision of biologically relevant substrates and selective enhancement of keratinocyte growth by manipulation of the medium or incubating conditions. A series of disparate techniques, many of which combine these elements, appears to be emerging for keratinocyte culture, each yielding cells closely resembling the parent tissue.

2.2.1 Feeder Cell Supports

One of the first reported methods of epithelial cell culture was pioneered by Rheinwald and Green (1975) and is

still widely used today. They achieved the successful clonal growth of human keratinocytes from newborn foreskin and a murine teratoma cell line (XB) on lethally irradiated feeder layers of Swiss 3T3 cells. These feeder cells largely suppress the growth of fibroblasts and remaining contaminants can be removed by brief exposure in 0.2% EDTA. Colony formation of human keratinocytes was found to be entirely dependent on the presence of 3T3 cells although the teratoma XB line could form colonies but not keratinize if seeded at a high enough density in the absence of 3T3 cells. Replacement of feeder cells by 3T3 conditioned medium was also able to support the growth of the XB line, but human keratinocytes failed even to initiate colonies. The authors concluded that the interaction of the two cell types was essential for the development of a keratinising epithelium. With the XB line, conditioned medium which had been diluted to less than 20% of the incubating medium, showed reduced effectiveness. It was thought possible that the removal of inhibitors by fibroblasts was involved in the conditioning, since simple dilution of growth factors seemed unlikely.

More recently, Eisinger et al (1979) have shown that human epidermis is not dependent on the presence of feeder cell layers, collagenised substrates, or special nutrients providing conditions of seeding density, pH and temperature are optimised. They grew over 200 specimens from eight anatomical sites with seeding densities of 2.5×10^5 cells cm^{-2} in acidic medium (pH 5.6 - 5.8) at 35 - 37°C. They found, however, that under these conditions only very limited

passage was possible. A maximum of 5 transfers was achieved for foreskin keratinocytes, but less so for other keratinocytes without the addition of substances which raise intracellular cAMP. Seeding densities used by this group were also exceptionally high and may be a limiting factor for other laboratories. Further problems may also arise in the overgrowth of keratinocyte colonies by fibroblasts at late passage in the absence of feeder cell cover.

Lechner et al (1980), for example found that bronchial epithelial cells still require a feeder layer even in the presence of additional growth factors in the medium. Interestingly too, while Lechner et al (1980) found, in agreement with Rheinwald and Green (1975), that cell lines of mesodermal origin provided the most favourable support for epithelial cells, Stampfer et al (1980) found that conditioned media from other epithelial cell lines were most effective at promoting keratinocyte growth from normal human mammary tissues. The ability of several cell types to serve as feeder layers for breast and cervical epithelia has also been noted by Stanley and Parkinson (1979). These may reflect genuine differences in epithelia derived from different sites. Despite progression towards identifying essential factors involved in keratinocyte growth, serial subcultivation in the absence of 3T3 or other cellular support has proved very difficult. It seems certain that the provision of a biologically relevant substrate such as fibronectin, extra cellular matrix or collagen appears to

increase plating efficiency. These substrates both improve cellular attachment (Macaig et al 1981) and may reduce the serum requirment for clonal growth (Ham and McKeehan 1979; Lechner et al 1980). Lillie et al (1980) also found that growth of rat lingual epithelial cells on collagen rafts which draw up medium by capillary action, show an increase in the degree of tissue organisation, and synchronous rather than sporadic keratinisation of the upper layers. The mircoenvironment, so often overlooked, may be much more important than previously thought.

2.2.2 Optimisation of Media for Keratinocyte Growth

Attempts to improve media for keratinocyte growth which would obviate the need for the laborious preparation of feeder layers were based on two considerations. Firstly, to define precisely the factors (including those present in serum) necessary for keratinocyte growth and secondly, to exclude the possible inductive effects of underlying mesenchyme when investigating epithelial cell functions in culture.

In modifying commercially available media, which had hitherto been developed primarily for fibroblasts, investigators had to bear in mind that, while enhancing keratinocyte growth, the medium had to select against fibroblasts. One of the simplest ways to achieve this was the addition to commonly used media of compounds which would specifically inhibit fibroblasts. Sporadic reports of this nature have appeared in the literature.

2.2.3 Fibroblast Inhibition

The following methods have been reported to inhibit growth of fibroblasts.

2.2.3.1 Thimerosal

Braaten et al (1974) successfully used sodium ethylmercurithiosalicylate (thimerosal) for the specific removal of fibroblastoid cells in primary cultures of pancreas islets with no apparent effect on their endocrine function. Later, Wagner and Matthews (1975) used thimerosal to grow pure cultures of endothelial cells without apparent effects on endothelial cell function. The mechanisms of the toxic effects of thimerosal to fibroblastoid cells are unknown.

2.2.3.2 Spermine Tetrahydrochloride

More recently, Schuster et al (1985) reported the addition of spermine tetrahydrochloride to epithelial cell cultures derived from hamster cheek pouch. The enzyme amino oxidase, which is present in higher concentrations in fibroblasts than in epithelial cells, converts spermine to an unstable aldehyde and then to acrolein both of which are toxic. Both thimerosal and spermine, however, have one drawback in that they are also toxic, although less so, to non fibroblast cells.

2.2.3.3 Antibody Mediated Cytotoxicity

A more specific agent which selectively kills fibroblasts has been developed by Gusterson et al (1981). They raised a monoclonal antibody against fibroblasts which kills these cells by complement mediated cytotoxicity. While this may be a promising method of fibroblast control, the antibody is not yet commercially available and may prove prohibitively expensive for routine tissue culture.

2.2.3.4 Temperature Sensitivity

A much simpler method for fibroblast control was adopted by Jensen and Therkelsen (1981). They reported that reduction in incubator temperature to 32 - 33°C was effective in inhibiting fibroblast growth with no effect on the growth rate of human foreskin epithelial cells. Indeed, Marcelo et al (1978) subsequently found that lower temperatures actually increase the in vitro lifespan of fully stratified epithelial cell cultures with no apparent decrease in proliferative activity. Recently, Arneholt-Bindslev et al (1987), reported that the optimum growth temperature for human oral epithelium was 34°C.

Other reports, however, (Hawley-Nelson et al 1980), have shown that reductions in temperature to 34°C or 31°C reduced colony numbers respectively to 50% and 10% of that of the controls. Eisinger et al (1979) also reported that reducing the incubating temperature below 35°C reduced colony forming efficiency of keratinocytes. The issue remains

unresolved.

2.2.4 Epithelial Cell Enhancement

Latterly, attempts have been made to specifically develop media for keratinocytes which allow plating in the absence of feeder suppport at low seeding densities and which increase their capacity for serial subcultivation. In a number of laboratories detailed studies of the conditions which favour keratinocytes have yielded interesting results. Fortunately, the requirements of epithelial cells differ sufficiently from those of fibroblasts to inhibit overgrowth by fibroblasts in such selective media.

The assessment of growth promoting additives, however, is complicated by a number of factors. The composition of the basic medium markedly influences the serum and growth factors required and this has been extensively reviewed by Barnes and Sato (1980). A further complication in assessing growth additives arises from the use of tissue from different sources. The most common sources of keratinocytes for these studies are newborn foreskin, oral mucosa, breast epithelium and in some laboratories transformed epithelial cell lines. The cells from each of these sources respond differently to growth additives. Indeed, three established epithelial breast lines (MCF-7, MDA-231 and ZR-75-1) differ markedly in their nutrient requirements and these requirements differ again from those necessary for the primary culture of normal breast epithelium (Osborne et al 1980; Allegra and Lippman,

1978; Anderson and Buerhing, 1983). Both MCF-7 and ZR-75-1 require insulin and transferrin but the basal medium and other additives are different. MDA-231, unlike MCF-7 cells, grown in the same medium were found to be completely unresponsive to insulin or EGF and it may be that only certain tumours retain biomolecular capacity to respond to these hormones.

In addition, few reports take into account possible synergistic effects of additives or fully investigate dose dependent relationships of these growth factors confirmed by Allegra and Lippman (1978), Mather and Sato (1979), Hayashi et al (1978), Peehl and Ham (1980a,b) and Tsao et al (1982). Allegra and Lippman (1978) showed clearly the biphasic dose response of the breast cell line ZR-75-1 to some hormones. Triiodothyronine (T3), for example, stimulated this cell line at concentrations as low as 1.0 nM to 0.1 uM but the effect on growth was diminished at higher concentrations (10 uM) and eventually became inhibitory. Optimal concentrations of hormones may also change as the medium becomes more defined. Wu and Sato (1978) found that critical components of the medium for HeLa cells are hydrocortisone (HC) and EGF. Aldosterone can, however, replace hydrocortisone and if the basal medium is replaced by the more intricate medium MCDB 105 then the need for hydrocortisone, insulin and EGF are eliminated. The fact that the effects of some hormones can be mimicked by manipulation of the low molecular weight nutrients leads to speculation that some hormones act in cell

culture by regulating the balance of nutrients inside the cell (Holley, 1972). Interpretation of the requirements of a particular tissue or the results of growth studies therefore requires careful consideration.

Workers in Ham's laboratory have studied in great detail the growth requirements of keratinocytes from human epidermis and have succeeded in producing a medium which contains no added undefined supplements and which supports clonal growth at low inoculum densities. They originally found that the need for a feeder layer or conditioned medium could be eliminated by the use of medium M199 which was supplemented by pituitary extracts (PE), hydrocortisone and 20% foetal bovine serum (FBS) (Peehl and Ham, 1980a). Subsequently, Peehl and Ham (1980b) found that the medium F12 eliminated the need for pituitary extract and permitted the use of dialysed foetal bovine serum proteins (FBSP) in place of whole FBS. Optimisation of the medium composition for keratinocytes resulted in a new medium designated MCDB 151 and Peehl and Ham (1980b) found, in this medium, they could further reduce the levels of FBSP to less than 1 mg ml^{-1} and achieve clonal growth. The low levels of calcium (30 uM) and the higher levels of adenine seemed to be of particular importance in the medium. Calcium shifted the balance between multiplication and terminal differentiation in favour of replication and this has been confirmed by Hennings et al (1980) and Boyce and Ham (1983).

In a later study Tsao, Walthall and Ham (1982) replaced the FBSP in the medium with a precisely defined

mixture of nutrients and further reductions in the size of the cellular inoculum needed were achieved. Tsao et al (1982) had previously found that bovine pituitary extract had a sparing effect on the amount of FBSP required for the multiplication of keratinocytes and a reduction in the amount of hydrocortisone was required at low inoculum densities. Trace elements, particularly selenium, were also essential in media with low amounts of total protein. None of the trace elements were inhibitory and therefore they were included in all their media thereafter. In the complete absence of FBSP, it was found that keratinocytes still responded to insulin, transferrin and hydrocortisone, and EGF was once again required although the optimal concentration had shifted. The concentration previously used was inhibitory and was subsequently lowered. The last undefined supplement was pituitary extract and this was finally replaced with phosphoethanolamine (PEtn) which was found to be the active ingredient of pituitary extract by Kano-Sueoka et al (1979). Although this new medium (MCDB 152) could support clonal growth of keratinocytes in the absence of any undefined supplements successful passage was not reported.

In 1983, however, Boyce and Ham first reported the use of a new medium MCDB 153 which differed from MCDB 152 in its concentrations of $FeSO_4$ and $ZnSO_4$. With the addition of low levels of EGF, IS, ethanolamine (Etn), PEtn, and wBPE, they could serially passage foreskin keratinocytes between 5 - 7 times with a cellular inoculum of only 400 cells cm^{-2}

compared with Eisinger's required inoculum of 2.5 x 10^5 cells cm^{-2} in a simpler medium.

It remains to be seen whether keratinocytes from other sources can also be grown at clonal densities in medium MCDB 153 or whether epithelia from different sources will require other additives; for example, whether breast epithelium will continue to require the addition of steroid hormones such as oestradiol and prolactin (Anderson and Buerhing, 1983). It seems likely, that as with other cell types, several media will eventually become available for routine keratinocyte culture.

2.3 SUBCULTURE

When cells become a confluent monolayer in primary culture they can be detached from the Petri dish by trypsinisation and subcultured (or passaged) either into a larger vessel, or by plating half the original number of cells into a dish of the same area i.e. a 1:2 split ratio. The cells undergo further division and the primary culture becomes a cell line. With each successive subculture the cells which proliferate most rapidly and which can withstand the rigours of subculture procedures gradually predominate.

2.3.1 Ageing or Differentiation

Normal human fibroblasts continue to grow *in vitro* through a limited number of cell generations but will eventually die out. The number of population doublings (usually 20-80) depends on the cell strain and the culture

conditions but is consistent for a given cell line grown in similar conditions. This reproducibility of culture lifetime may be analagous to ageing *in vivo*. Ageing of cells *in vitro* is described as a reduced proliferative capacity that evolves as a function of serial passages or time in culture (Schneider and Mitsui, 1976 ; Cristofalo, 1970; Gelfant and Smith, 1972). An alternative postulated by Bell *et al* (1978) suggests that the phenomenon of ageing i.e. cessation of proliferation of diploid cells in culture, represents a step of differentiation and not senescence. They found no causal relationship between cell divisions and ageing. From cinematographic histories of cells, and analysis of their lineages, they found that cells were not required to pass through a fixed number of divisions before leaving the cell cycle. Nor did they find that non cycling cells were moribund. Cultures could be kept alive for up to 14 months after division had ceased. They proposed that populations of cells in culture consisted of both cycling and differentiated cells. At low passage, these cells would be mostly cycling, whereas at high passage there would be an increasing population of differentiated cells. Thus when the number of differentiated cells is greater than the number arising from cell division, then further division of the cultures in a 1:2 split ratio, or greater, results in an overall decline in cell numbers.

Cristofalo (1970) argues, however, that cells which become non-dividers lose the ability to synthesize the proteins necessary for initiation of DNA synthesis or

activation of some other aspects of the cell cycle. *In vitro* therefore, as more cells lose their ability to synthesize these proteins, the culture will eventually senesce and die out. Support for these arguments arises from evidence that growth factors and hormones have been found to prolong the post-mitotic state of human cells in culture and may also reverse age dependent changes in cellular metabolism (Leith, 1967; Yuan and Chang, 1969; Cristofalo, 1970; Rheinwald and Green, 1975). Doubtless in answering the question of whether cells age or merely differentiate *in vitro*, a compromise between these opposing stances will be found.

Continuous or immortal cell lines may arise spontaneously in some normal murine cultures but generally only occur in normal human cell lines after treatment with carcinogenic chemicals or oncogenic viruses. This process is termed transformation. Transformed cells undergo morphological and kinetic alterations, some properties of which are associated with features of malignant cells, for example an unrestricted capacity for cell division. Again within Bell's framework, the difference between transformed and normal diploid cells in culture is the inability of transformed cells to differentiate rather than some intrinsic capacity not to age.

2.3.2 Serial Subculture of Keratinocytes

The passage of epithelial cells, like their routine growth, has proved much more difficult than the subculture of

fibroblasts. Keratinocytes *in vivo* not only divide to give rise to progeny, but a proportion of these cells move from the basal layer where division occurs and terminally differentiate to form the protective layers of skin which are continually shed and replaced. Differentiation of fibroblasts *in vivo* is seen only in specific circumstances such as the granulation tissue of a wound.

At least, partly because of the commitment to terminal differentiation, colony forming efficiency of epithelial cells on transfer is lower than that of fibroblasts. Other factors may also contribute such as loss of three dimensional cellular organisation in culture, the loss of suitable cell-cell interaction and the absence of necessary nutritional factors.

Indeed, the role of growth factors and steroid hormones in maintaining cells in a cycling state has been noted in several culture systems. Leith (1967) proposed that the addition of hydrocortisone to cell cultures may retard senescence of cultures by derepression of RNA synthesis. Although the mechanism of derepression is unclear, addition of glucocorticoids to several cell culture systems has been noted to induce formation of specific proteins e.g. alkaline phosphatase and tyrosine transaminases (Cox and MacLeod, 1962; Pitot *et al* 1964). Griffin and Ber (1969) further reported that alkaline phosphatase induction by hydrocortisone in HeLa cell culture occurred exclusively in the S phase of the cell cycle. If Cristofalo's (1970) proposed mechanism for the existence of a division initiating

protein is correct then dividing cells passing through G1 to S phase of the cell cycle can be rescued from producing non dividing daughter cells.

Later, Rheinwald and Green (1977) found when EGF was added to epithelial cultures exponential growth was maintained for longer. In the absence of EGF between 6-7 transfers were achieved while in the presence of EGF 16-17 transfers were common. Investigation of the mode of action of EGF showed that it increased colony forming ability before the growth rate was detectably altered. It was concluded that there must be a change in the state of the dividing cells, better enabling them to form new colonies i.e. an alteration in the generation time rather than an alteration in the relative magnitude of the dividing fraction.

Cell-cell interation may also be particularly important since, in the absence of mesenchyme support, there have been few reports of successful serial subculture. Eisinger et al (1979) reported the growth of epithelial cells from many sources in the absence of dermal components or medium supplements using high plating densities - 2.5×10^5 cells per cm^{-2}. With fewer than 1.0×10^5 cells per cm^{-2} confluence was not achieved. Cells either failed to grow or colonies failed to coalesce at this seeding density. Milo et al (1980) have also reported that routine subculture of newborn foreskin keratinocytes is possible in the absence of extrinsic factors in the medium and without the use of conditioned medium or cellular support but again with high

inoculum densities. Stampfer et al (1980) passaged epithelial cells from normal human mammary tissue, grown initially as organoids in culture, through a maximum of 4 transfers using conditioned medium from other epithelial cell lines.

Recently, using the considerably more complex medium, MCDB 153, Boyce and Ham (1983) have achieved serial subculture of normal human keratinocytes using inoculum densities of only 400 cells per cm^2. This report would seem to indicate that the presence of mesenchyme cells is not a critical requirement for keratinocyte culture at low clonal density but that the feeder effect, given enough investigation, can be provided directly by the medium.

Reports of subculture in the absence of extrinsic factors or mesenchyme support do, however, remain isolated and this may continue to be a problem that will hamper routine culture of normal epithelial cell lines for some time to come.

In this study a number of methods were investigated for growing human oral keratinocytes from gingival biopsies and from oral squamous cell carcinomas. A medium was developed which would support keratinocyte growth in vitro, and which prevented overgrowth of epithelial cultures by fibroblasts arising from the lamina propria. Populations of cultured keratinocytes were characterised by light and electron microscopy to determine their suitability for use in studies of fibrinolysis. Growth kinetics and karyotypic analyses

were also performed to study the nature of the cells in culture.

2.4 CULTURE OF SUSPENSIONS OF ORAL KERATINOCYTES ON 3T3 FEEDER LAYERS

2.4.1 Introduction

A number of methods have been developed for the growth of keratinocytes *in vitro*. Suspension cultures may be plated directly onto plastic or 3T3 feeder layers, and small pieces of explanted tissue may be grown successfully in culture. In order for cultures to achieve confluence, keratinocytes must be plated at high seeding densities (125,000 cells cm^{-2}) when they are grown directly on plastic (Eisinger, 1979) but this seeding density may be reduced (12,500 cells cm^{-2}) when grown on 3T3 feeder cells (Rheinwald and Green, 1975). This latter method is particularly useful to either greatly amplify numbers of cultured keratinocytes or, when the biopsies from which cultures are to be derived are small.

In the present study, the use of lethally treated 3T3 feeder cells for oral keratinocyte culture was investigated and cultures were treated with Mitomycin C to inhibit cell division before seeding with oral keratinocytes. It was necessary to first assess the dosage of Mitomycin C which would be required to inhibit cell growth, whilst allowing 3T3 cells to remain viable for several days. The cytotoxicity of Mitomycin C to subconfluent layers of 3T3 cells was determined using a chromium release assay (Wright

et al 1978). Cell proliferation was assessed by thymidine incorporation and cell viability was determined using trypan blue staining.

2.4.2 Materials and Methods

2.4.2.1 3T3 Feeder Cells

Swiss albino mouse embryo fibroblasts (CCL-92) were grown to approximately 75% confluence in the Glasgow Modification of Minimum Essential Medium (GMEM) as follows:

Glasgow Modification of Minimum Essential Medium (x 10 liquid)*	10%
Sodium bicarbonate	30 mM
L-Glutamine**	2.0 mM
Penicillin	100 Uml^{-1}
Streptomycin	100 $ugml^{-1}$
Newborn Bovine Serum	10%

*Unless otherwise stated all media components were obtained from Flow Laboratories.

**L-glutamine is omitted from all commercially available media supplied in liquid form and must be added before use.

2.4.2.2 Determination of Cytotoxicity of Mitomycin C to 3T3 Cells using a Chromium Release Assay

3T3 cells were seeded onto 96 well plates and grown to approximately 75% confluence. The cells were then washed in

serum free medium and incubated overnight in medium containing ^{51}Cr - aqueous sodium dichromate (sp.act 300 uCi/ugCr Amersham International) at a concentration of 1uCi per well. The cells were then washed three times in 600 ul of serum free medium and serial dilutions of Mitomycin C from 30 $ugml^{-1}$ to 3.75 $ugml^{-1}$ were added to the cells in a final volume of 100 ul of medium per well. The supernatants were harvested after 1h and counted in an LKB-Wallac 1214 gamma spectrophotometer. The percentage release of ^{51}Cr into the supernatant was calculated in the following way:

$$\% \text{ cytotoxicity} = \frac{\text{sample value} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

where spontaneous release was determined by incubating 3T3 cells in GMEM with 10% serum and maximum release by lysis of 3T3 cells in 1% sodium dodecyl sulphate (BDH Chemicals).

2.4.2.3 Incorporation of Tritiated Thymidine into Mytomycin C Treated 3T3 Cells

3T3 cells were plated into 12 well plates and grown to approximately 60-70% confluence. After treatment with serial dilutions of Mitomycin C from 30 $ugml^{-1}$ to 1.875 $ugml^{-1}$ for 1h at room temperature, the cells were washed three times in serum free medium and re-fed with GMEM containing 10% serum and incubated overnight at 37°C. The medium was then aspirated and the 3T3 cells were incubated with medium containing tritiated thymidine (sp.act. 5Ci/mMol, Amersham

International) at a concentration of 2uCi per well for five hours. The supernatants were aspirated and the cells washed three times in PBSA. The cells were lysed in 0.025M ammonium hydroxide for two hours on a rocking platform. To 0.5 ml of the lysate was added 4.5 ml of Optiphase Highsafe II scintillation fluid (Pharmacia-LKB) and the components mixed thoroughly on a whirlimixer. Tritiated thymidine incorporation into cells was determined by measuring the beta activity of samples in an LKB-Wallac 1218 liquid scintillation counter.

2.4.2.4 Growth and Viability of 3T3 Cells After Treatment with Mitomycin C

3T3 cells were seeded at a density of 10^5 cells ml^{-1} into 75 cm^2 flasks and allowed to plate down (about 2-4h). Cells were treated with Mitomcyin C at concentrations ranging from 30 $ugml^{-1}$ to 3.75 $ugml^{-1}$ for one hour and then the cells were washed three times in serum free medium before they were returned to their normal medium. After 3, 6 and 9 days, cells were trypsinised in 5 ml 0.25% trypsin and 0.02% EDTA. The trypsinised cell suspension was added to 5 ml of medium containing 10% serum and centrifuged for 5 min. at 200g. The supernatant was aspirated and the cells resuspended in 1 ml of medium. To 0.1 ml of cell suspension was added 0.1 ml of trypan blue stain and the cell numbers per flask determined using an improved Nebauer haemocytometer chamber. Cell viability was measured by the exclusion of trypan blue from viable cells.

2.4.2.5 Trypan Blue Stain

Trypan blue	400 mg
Sodium Chloride	810 mg
Potassium dihydrogen orthophosphate	60 mg

The mixture was dissolved in 95 ml of distilled water, boiled for 5 min. and allowed to cool. The solution was then filtered and the pH adjusted to pH 7.2 with 1M sodium hydroxide. The solution was made up to a final volume of 100 ml.

2.4.2.6 Preparation of Epithelial Cell Suspensions

Pieces of gingival mucosa obtained during routine wisdom tooth extractions at Edinburgh Dental Hospital were transported to the laboratory in GMEM on ice and were used within three hours of surgery. Using aseptic techniques, as much connective tissue as possible was removed from each sample and the remainder finely minced using scalpel blades. The tissue was placed in 0.25% collagenase:dispase (Boehringer:Mannheim) in calcium and magnesium free PBSA for 3-4 hours. The cell suspension was periodically decanted and fresh collagenase:dispase added. The cells were harvested by centrifugation at 200g for 10 min. and the cells seeded at a concentration of 2×10^4 cells cm^{-2} onto 3T3 feeder cells which had been treated with Mitomycin C (3.75 $ugml^{-1}$).

The tissues were placed in medium (see Section 2.4.2.7) and incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C.

2.4.2.7 Keratinocyte Medium for Suspension Cultures

Eagle's Minimum Essential Medium (x 10 liquid)	10%
L-glutamine	2.0 mM
Sodium bicarbonate	30 mM
Penicillin	100 Uml^{-1}
Streptomycin	100 $ugml^{-1}$
Human Serum	10%

2.4.2.8 Preparation of Human Serum

Human serum from one individual was used throughout these experiments. This was prepared by clotting whole blood (obtained by venepuncture) overnight at 4°C. The blood was then centrifuged at 500 g for 15 min. and the serum supernatant decanted. The serum was then heat inactivated by heating at 60°C for 30 min., was sterile filtered through a 0.22 um filter and stored at -20°C in 5 ml aliquots.

2.4.3 Results

2.4.3.1 Release of ^{51}Cr-Sodium Dichromate from Mitomycin C Treated 3T3 Cells

The percentage release of ^{51}Cr above background release was between 10 - 15% for concentrations of Mitomycin C below 7.5 $ugml^{-1}$ (Fig. 6). Above this concentration, the release of ^{51}Cr from cells increased from 19% at 15$ugml^{-1}$ to 35% at 30$ugml^{-1}$ of Mitomycin C. Toxicity of Mitomycin C, indicated

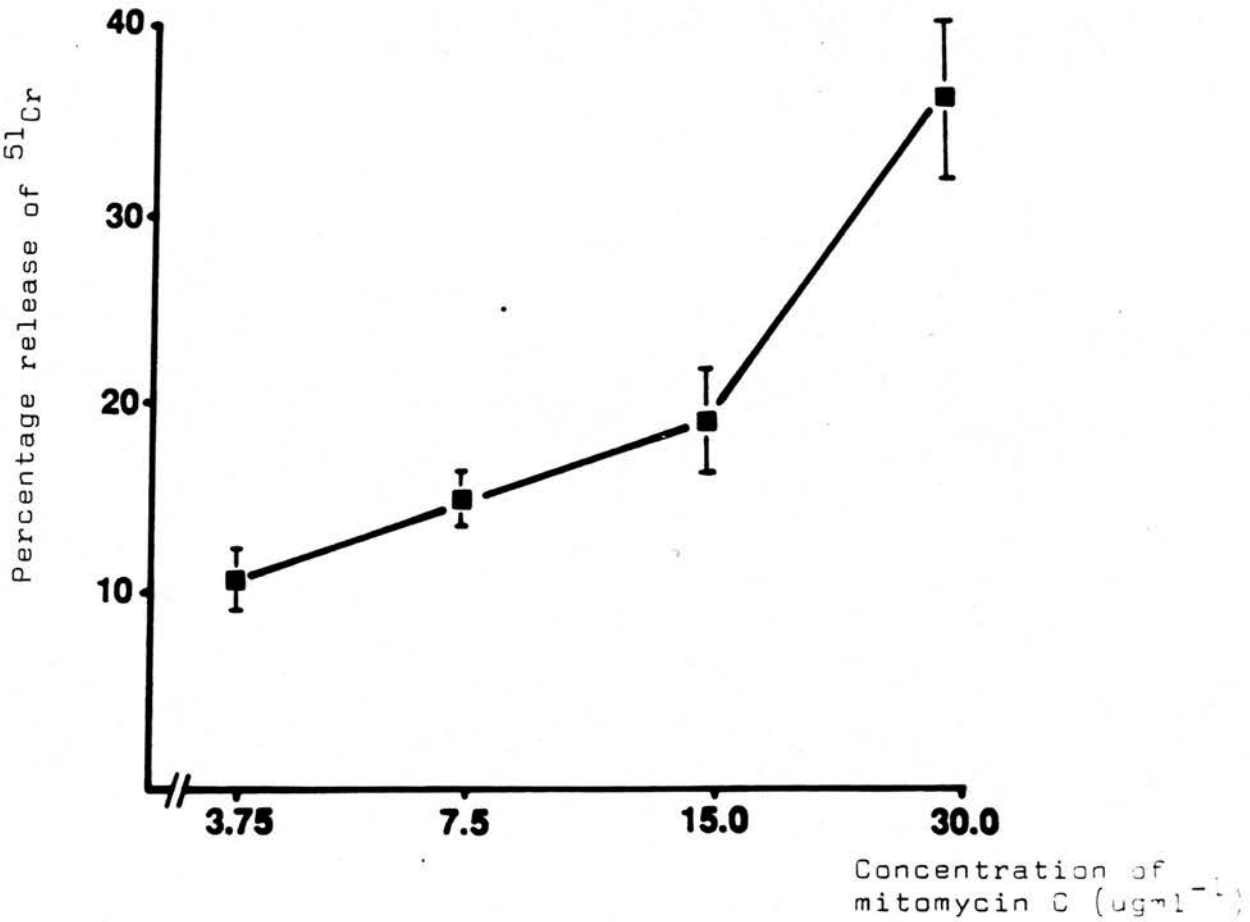


Fig. 6 Percentage release of ^{51}Cr sodium dichromate from Mitomycin C treated 3T3 cells.

by the release of chromium from the cells, was apparent at all concentrations of Mitomycin C but was greatly increased at concentrations above 7.5 $ugml^{-1}$.

2.4.3.2 Incorporation of Tritiated Thymidine by 3T3 Cells Treated with Mitomycin C

The results shown in Table 1 and Fig. 7 indicate that incorporation of tritiated thymidine one day after treatment of 3T3 cells with Mitomycin C decreased in a dose dependent manner. No concentration of Mitomycin C studied was sufficient to completely inhibit tritiated thymidine incorporation although cells treated with concentrations of Mitomycin C above 3.75 $ugml^{-1}$ never reached confluence even after 10 days in culture.

2.4.3.3 Cellular Proliferation and Viability After Treatment with Mitomycin C

The number of cells present in cultures after treatment with different concentrations of Mitomycin C is shown in Table 2 and Fig. 8. After nine days in culture a slight reduction in cell numbers was noted after treatment of 3T3 cells with 3.75$ugml^{-1}$ of Mitomycin C when compared to untreated cultures. With the lower concentration of Mitomycin C (1.875 $ugml^{-1}$) the number of cells present doubled over the culture period and was only slightly less than that in the untreated cultures.

Trypan blue exclusion staining of cells treated with

TABLE 1

INCORPORATION OF TRITIATED THYMIDINE BY MITOMYCIN C TREATED 3T3 CELLS

Concentration Mitomycin C (ug ml^{-1})	Disintegrations per minute (d.p.m.) ±S.D.
0	670,672 ±59,342*
1.875	332,866 ±23,116
3.75	275,580 ±21,736
7.5	216,685 ± 8,808
15.0	171,773 ±15,082
30.0	133,275 ±10,798

*Each value is a mean of four replicates

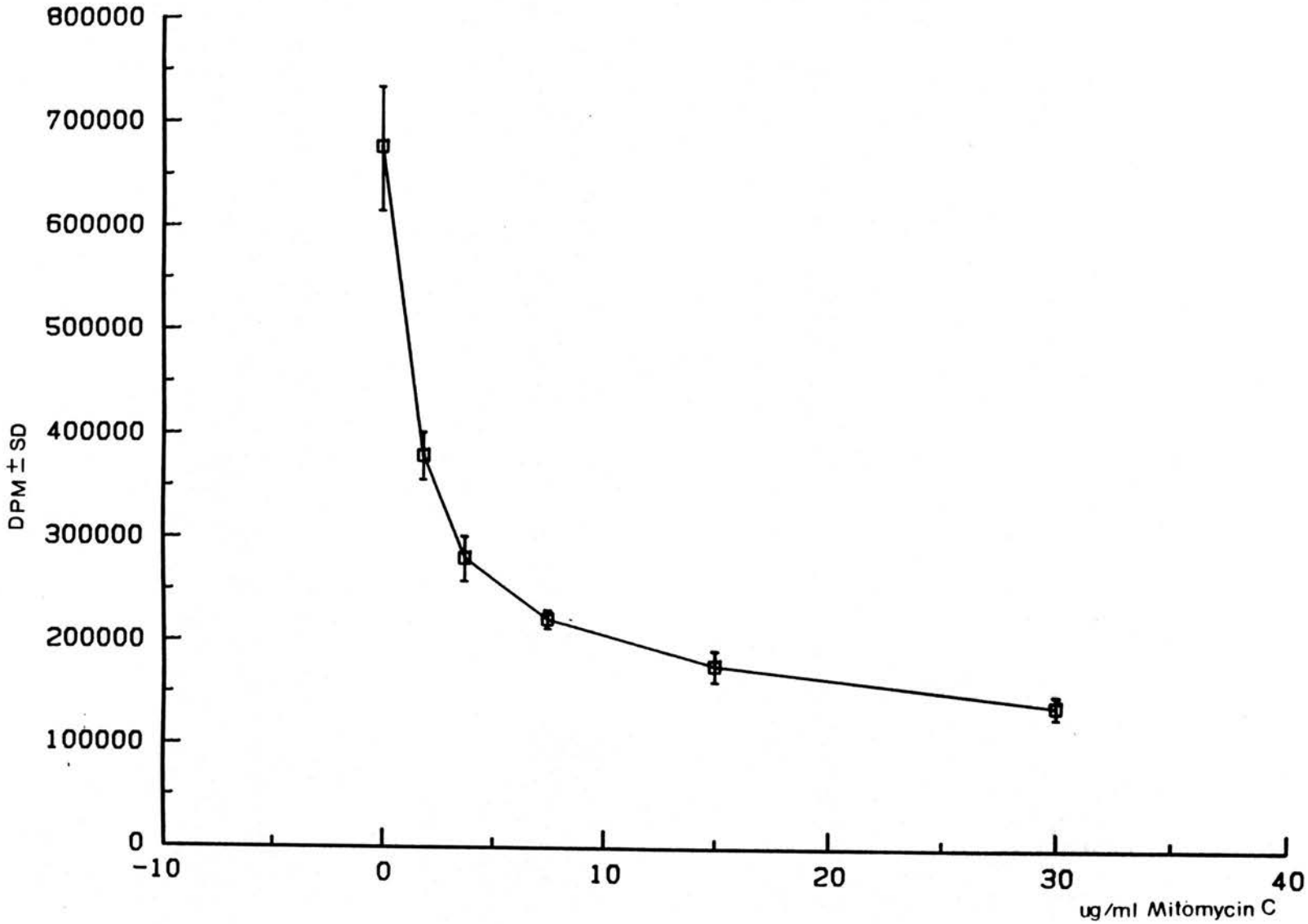


Fig. 7 Incorporation of tritiated thymidine by Mitomycin C treated 3T3 cells.

TABLE 2

REPLICATION OF 3T3 CELLS AFTER TREATMENT WITH MITOMYCIN C* (CELLS $ml^{-1} \times 10^5$)

Concentration Mitomycin C (ug ml^{-1})	Days after plating		
	3	6	9
0	2.0	2.6	2.8
1.875	1.75	2.2	2.35
3.75	1.3	1.1	0.9

Cells were plated at a seeding density of 10^5 cells ml^{-1}

*Each value is a mean of three replicates

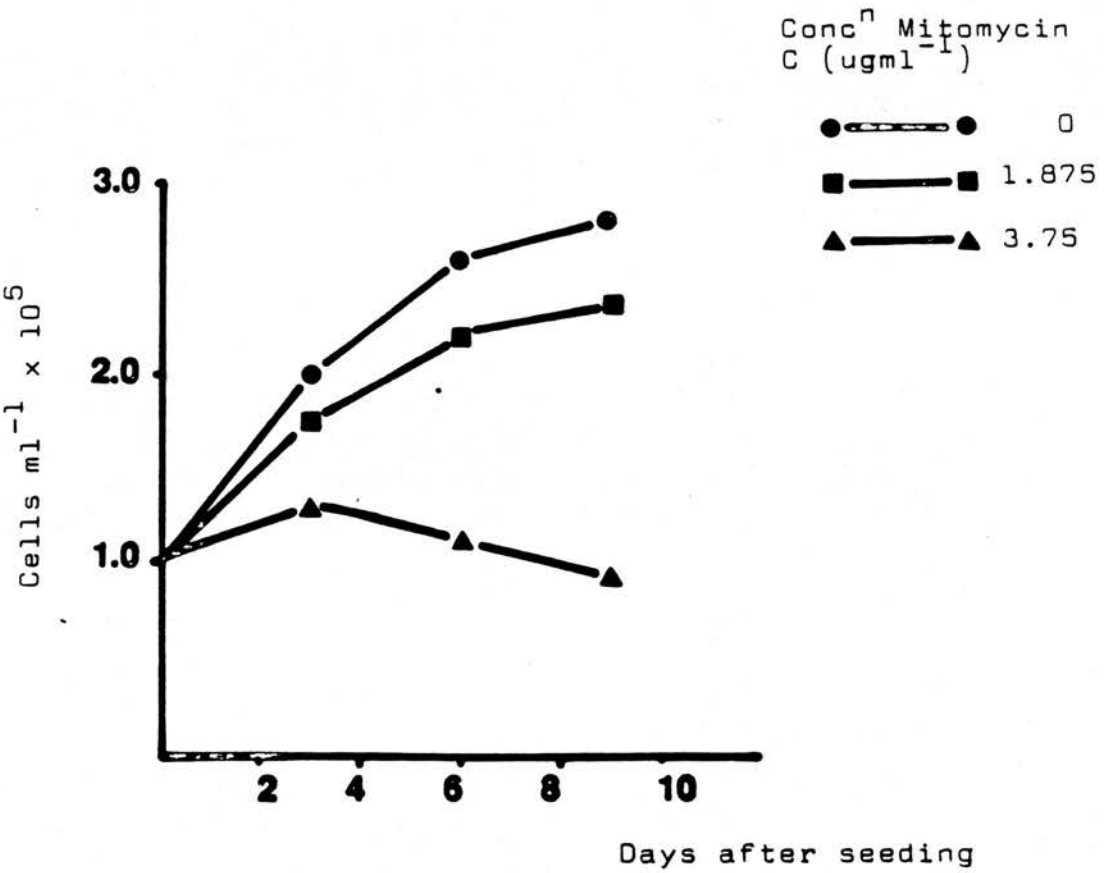


FIG. 8

Replication of 3T3 cells treated with mitomycin C.

Mitomcyin C was studied two and eight days after treatment. The data in Table 3 indicate that even after treatment with 30 $ugml^{-1}$ of Mitomycin C over 85% of the cells remained viable after two days compared with over 90% in control cultures. After eight days of culture, however, the percentage of viable cells recovered after treatment with concentrations of Mitomycin C above 7.5$ugml^{-1}$ had fallen to less than 60% compared with 86% in untreated cultures. In cultures treated with 3.75$ugml^{-1}$ Mitomycin C, which was sufficient to inhibit cellular proliferation, viability of cells after eight days was just under 70%.

2.4.3.4 Growth of Oral Keratinocyte Cell Suspensions on 3T3 Feeder Cells

Tissue samples obtained from wisdom tooth extractions were generally small and, after treatment of biopsies with collagenase:dispase to obtain single cell suspensions harvests were usually between 5×10^4 and 5×10^5 cells. Keratinocytes seeded onto Mitomycin C treated feeder cells plated down and after several days in culture clones of epithelial cells were apparent amongst the 3T3 cells (Fig. 9). Of necessity, seeding densities were low, usually in the order of 2×10^4 cells cm^{-2} which resulted in clones failing to maintain proliferation long enough for cells to coalesce and grow to confluence before senescing.

TABLE 3

PERCENTAGE VIABILITY OF 3T3 CELLS AFTER TREATMENT WITH MITOMYCIN C

Concentration Mitomycin C (ug ml^{-1})	Viability	
	2 days	8 days
30	88.7 (370)*	57.3 (293)
15	95.4 (214)	52.1 (301)
7.5	88.5 (570)	57.6 (189)
3.75	90.1 (546)	68.0 (276)
0	93.1 (372)	86.6 (411)

*Figures in parenthesis are numbers of cells counted

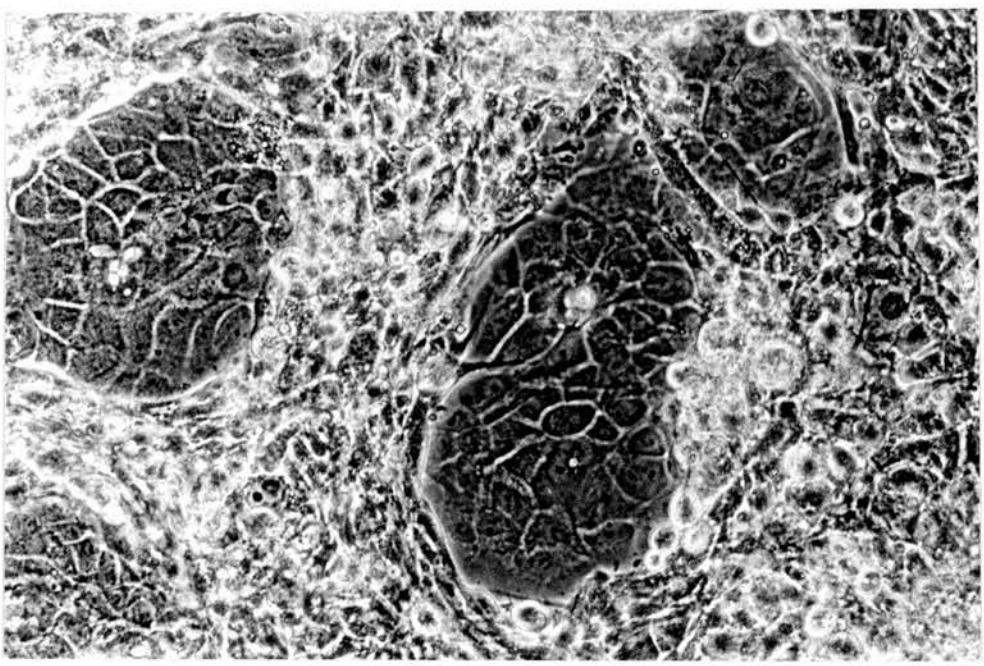


Fig. 9 Clones of epithelial cells growing on Mitomycin C treated 3T3 cells (x400 mag).

Conclusion:

With the techniques used in this study, oral keratinocyte suspensions were unlikely to provide sufficient cells for subsequent experiments. The use of explant culture was therefore investigated.

2.5 EXPLANT CULTURE OF ORAL EPITHELIUM

2.5.1 Introduction

There are a number of reports in the literature on the growth of oral keratinocytes in culture and the majority of these have relied on the use of explant cultures (Mlinek and Buchner, 1975; Gusterson and Monaghan, 1979; Arneholt-Bindslev *et al* 1987). The problem of overgrowth of epithelial cultures by fibroblasts is well recognised and approaches to minimising fibroblast contamination have included brief exposure to trypsin or EDTA (Owens *et al* 1971; Milo *et al* 1980; Price *et al* 1980), addition of chemicals which are toxic to fibroblasts to the media (Wagner and Matthews, 1975; Schuster *et al* 1984), alteration in incubation temperature (Jensen and Therkelsen 1981; Arneholt-Bindslev *et al* 1987), and the use of selective media (Macaig *et al* 1981; Tsao *et al* 1982; Boyce and Ham 1983). In the present study, a number of methods were investigated to control fibroblast contamination in explant culture. The possibility of passage of oral keratinocytes grown in explant culture was also investigated.

2.5.2 Material and Methods

2.5.2.1 Explant Culture

Pieces of gingival mucosa were trimmed to remove as much connective tissue as possible. Tissue was chopped into fragments approximately 1 mm^3 and placed epithelial side up in culture dishes. Eight to ten fragments per dish were anchored in place using sterile glass coverslips. The cultures were grown in MEM supplemented with L-glutamine (2.0mM), sodium bicarbonate (30mM), penicillin (100 Uml^{-1}), streptomycin (100 $ugml^{-1}$) and human serum (10%) (see Section 2.4.2.7).

2.5.2.2 Removal of Fibroblasts from Keratinocyte Cultures

Trypsin/EDTA

Explant cultures of keratinocytes grown for 2 weeks *in vitro* were washed in calcium and magnesium free PBSA and treated briefly with either 0.1% trypsin or 0.2% EDTA. Cultures were monitored under the microscope until fibroblast removal was complete. The treated cultures were then washed twice in serum free medium before re-feeding.

Thimerosal

Cultures were established and after one week the cultures were incubated in medium containing 0.5 - 1.5 $ugml^{-1}$ thimerosal for a further two weeks.

Cytotoxic antifibroblast monoclonal antibody

A cytotoxic monoclonal antifibroblast antibody

(LICR/LON FIB 86) was kindly provided by Dr. B. Gusterson, Ludwig Institute of Cancer Research, London. The antibody was prepared in the form of ascitic fluid. The production of the antibody used in these experiments has been described elsewhere (Edwards et al 1980). The antibody belongs to the subclass gamma-2b and was raised to fibroblasts derived from normal human breast using the hybridoma system of Milstein et al (1979).

Explant keratinocyte cultures, after 1 - 2 weeks in vitro, were washed twice in serum free medium. The antibody was added to each culture dish in 2 ml MEM at a final dilution of 1:400 and incubated at room temperature for 15 - 30 min. Antibody was aspirated and the cultures washed twice in serum free medium to remove any remaining antibody. Whole rabbit serum, as a source of complement, was added at a dilution of 1:20 and incubated for 1h at 37°C. Cultures were washed and returned to their normal medium and cultured for a further two weeks. Control cultures were treated with antibody or complement alone.

2.5.3 Results

2.5.3.1 Removal of Fibroblasts from Keratinocyte Cultures

Trypsin/EDTA

Removal of fibroblasts from mixed cultures by exposure to EDTA was generally found to be unsuccessful. However, brief trypsinisation of cultures with 0.1% trypsin was effective if fibroblast numbers were low and fibroblasts were

not covered by large epithelial sheets growing out from the explant. Repeated trypsinisation resulted in some epithelial detachment and damage.

Thimerosal

The addition of thimerosal to epithelial cultures at a concentration of $1.5ugml^{-1}$ was found to be toxic to epithelial cells on the basis of their morphology in the microscope and their slower growth rate. Further dilution of thimerosal to $0.5ugml^{-1}$, which was not toxic to epithelial cells, did not inhibit fibroblast growth.

Cytotoxic monoclonal antibody

This method was found to be successful in the present tissue culture system only if fibroblast contamination remained low. Treatment of cultures with cytotoxic antibody was far less effective in cultures heavily populated with fibroblasts. In control cultures treated with antibody or complement alone no removal of fibroblasts was observed. Cytotoxic antibody often failed to eliminate fibroblasts in a single treatment. The problem of access of antibody to fibroblasts growing underneath epithelial outgrowths was similar to that encountered with trypsin removal of fibroblasts.

Conclusion:

The failure of these simple measures to reduce fibroblast contamination in epithelial cultures required the

investigation of a selective medium to control fibroblast contamination in these explant cultures.

2.6 THE USE OF A SELECTIVE MEDIUM FOR GROWTH OF HUMAN ORAL KERATINOCYTES

2.6.1 Introduction

Refinement of selective media for cells in culture is for the most part labour intensive. The interaction of growth factors, trace elements and other additives requires the assessment of their individual contribution in basal medium and their synergistic or inhibitory effects with the addition of each defined factor. Each new addition or deletion to the medium should therefore be tested in a range of concentrations.

In this study, a basal medium was sought which, with minimal additions, would support keratinocyte growth but be selective enough to inhibit fibroblasts. In order to control the growth of fibroblasts in mixed cultures, the serum content of the medium was reduced and growth factors which would favour keratinocyte growth added. Five per cent serum normally provides the majority of cells with adequate levels of the B vitamins, other fat soluble vitamins and essential organic compounds, so no further additions of this nature were made.

Two further supplements were made to the medium. Non-essential amino acids which can normally be synthesized by cells *in vitro*, were added to the culture medium as these are

readily lost from the cytoplasm if the transmembrane concentration gradient is large. Sodium pyruvate was also provided as an additional carbon source to the cells. This is a common addition to commercially produced media e.g. Ham's F12, which is designed for culturing cells at low density. Unlike phosphorylated intermediates of glycolysis, it is readily lost from the cytoplasm to the external medium if the concentration gradient across the cell membrane is large. The inclusion of pyruvate in the medium also enables the cells to increase their endogenous production of carbon dioxide making them less dependent on the exogenous provision of carbon dioxide and bicarbonate (Leibovitz, 1963).

The medium was originally buffered in the normal way with 30mM sodium bicarbonate and by buffering in the gas phase with 5% carbon dioxide. Bicarbonate has poor buffering capacity at physiological pH but has low toxicity and a nutritional benefit to the culture. An additional buffer, Hepes, which has stronger buffering capacity at pH 7.2 - 7.6, was added to the medium in the presence of lowered amounts of sodium bicarbonate. Exogenous carbon dioxide was still supplied to prevent additional loss of dissolved carbon dioxide from the medium.

The effect of altering culture conditions, particularly the reduction in serum content and the addition of growth factors, was examined by measuring growth rates in primary cultures. Some authors have reported markedly inhibitory effects of antibiotics, particularly fungizone (amphotericin

B) towards epithelial cells in culture (Milo et al 1980). The effect of the removal of antibiotics from the culture medium was also investigated.

The use of primary cultures meant that different media could only be compared within cultures from an individual biopsy specimen.

2.6.2 Materials and Methods

2.6.2.1 Explant Culture

Specimens of gingival mucosa (>200) and specimens of oral squamous cell carcinomas (35) were set up in explant culture as previously described in section 2.5.2 and grown in a number of media.

Explanted tissues were incubated in a basal medium of MEM containing L-glutamine (2.0mM), sodium pyruvate (1.0mM), sodium bicarbonate (10mM), Hepes buffer (20mM), non-essential and amino acids (1%) antibiotics penicillin ($100Uml^{-1}$), streptomycin ($100ugml^{-1}$) and fungizone ($2.5ugml^{-1}$). In a series of experiments the effects of varying concentrations of serum and the addition of growth factors were assessed using the following series of media:

(a) basal medium + 10% human serum

(b) basal medium + 5% human serum + $10ngml^{-1}$ EGF

(c) basal medium + 10% human serum + $10ngml^{-1}$ EGF

(d) basal medium + 10% human serum + $10ngml^{-1}$ EGF + $5ugml^{-1}$ insulin

(e) basal medium + 5% human serum + $10ngml^{-1}$ EGF + 0.1mM

phosphoethanolamine + 5ugml^{-1} insulin

(f) basal medium + 5% human serum + 10ngml^{-1} EGF containing no antibiotics

The number of explants which attached and produced epithelial outgrowths was measured, and the areas of culture dishes covered by epithelium measured using a combination of image analysis and point counting (Dunnill 1698). This is fully described in Chapter 3 section 3.2.4.

2.6.2.2 Subculture

Near confluent cultures of oral keratinocytes were trypsinised in 2ml 0.25% trypsin/0.02% EDTA for up to 15 minutes. Medium (6 ml) containing 10% serum was added to the trypsin/EDTA solution and the cells centrifuged at 200g for 5 min. The supernatant was aspirated and the cells resuspended in 1 ml of medium containing 5% serum. The cells were passaged in a 1:1.5 - 1:2 split ratio.

2.6.3 Results

2.6.3.1 Growth of Gingival Oral Keratinocytes in Explant Culture

Of the explants grown in medium containing 10% serum, 76% of the explants produced epithelial outgrowth compared to 62% in medium containing 5% serum and EGF (Table 4). The provision of 5% serum appeared to be adequate for attachment of explants, although the number of explants attaching and producing outgrowth was slightly reduced. However, the areas

TABLE 4

THE PERCENTAGE OF EXPLANTS PRODUCING EPITHELIAL OUTGROWTHS IN MEDIUM CONTAINING EITHER 10% SERUM OR 5% SERUM AND EGF

Biopsy	Medium + 10% Serum	Medium + 5% Serum + EGF
1.	54.1	22.2
2.	95.7	83.0
3.	71.5	68.4
4.	83.6	70.2

of epithelial colonies in medium containing 5% serum and EGF were consistently larger than those grown in medium containing 10% serum (Table 5, Fig. 10).

Growth of epithelium in either of the above media appeared to be sub-optimal. In medium containing 10% serum supplement with EGF, a higher proportion of explants showed epithelial growth (67%) than those in medium supplemented with 10% serum only (61%) (Table 6). The areas of outgrowth in EGF containing medium were also larger in two of the three patient samples tested (Table 7, Fig. 10).

Addition of insulin to medium containing 5% serum and EGF resulted in improved growth rates in epithelial explants, but these differences were small (Table 8, Fig. 11). A further addition of phosphoethanolamine (Petn) to this medium actually resulted in inhibition of epithelial growth when compared to explants grown in medium containing 5% serum only (Table 9, Fig. 11). Growth of epithelium in medium supplemented with 5% human serum and EGF appeared to support good growth of epithelium. In medium containing 10% serum, fibroblast contamination was always noted.

The growth of oral keratinocytes in medium containing 5% serum, from which antibiotics were removed at the first feeding was also investigated. It was found that in nearly all cases the medium became contaminated, usually with fungus, after several days in antibiotic free medium. In those few cultures which remained uncontaminated and in which areas of epithelial outgrowths were measured, it was found

TABLE 5

AREAS OF EPITHELIAL COLONIES (mm^2) GROWN IN MEDIUM CONTAINING EITHER 10% SERUM OR 5% SERUM AND EGF AFTER 7 DAYS IN VITRO

Biopsy	Medium + 5% Serum + EGF	Medium + 10% Serum
1.	22.3 ± 3.7	10.1 ± 0.2
2.	84.6 ±12.0	44.6 ±16.6
3.	73.9 ± 8.4	40.2 ±12.1

n=52

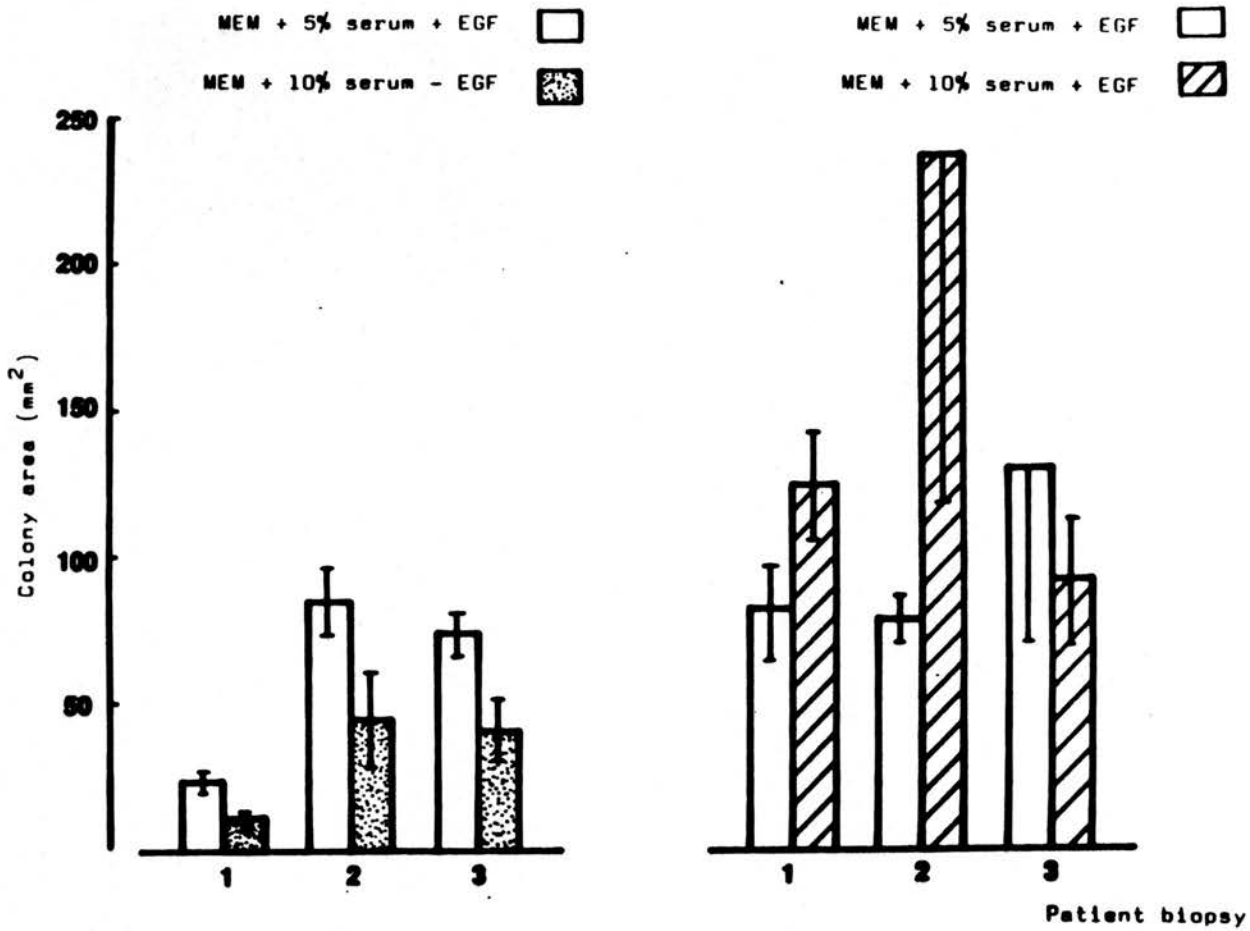


Fig.10 Areas of epithelial colonies (mm^2 ± S.E.) grown in various media.

TABLE 6

THE PERCENTAGE OF EXPLANTS PRODUCING EPITHELIAL COLONIES IN MEDIUM CONTAINING 5% OR 10% SERUM WITH EGF

Biopsy	Medium + 5% Serum + EGF	Medium + 10% Serum + EGF
1.	87.5	93.8
2.	63.6	83.3
3.	26.6	37.4
4.	76.9	72.2
5.	73.9	72.2
6.	71.4	73.3
7.	28.6	35.0

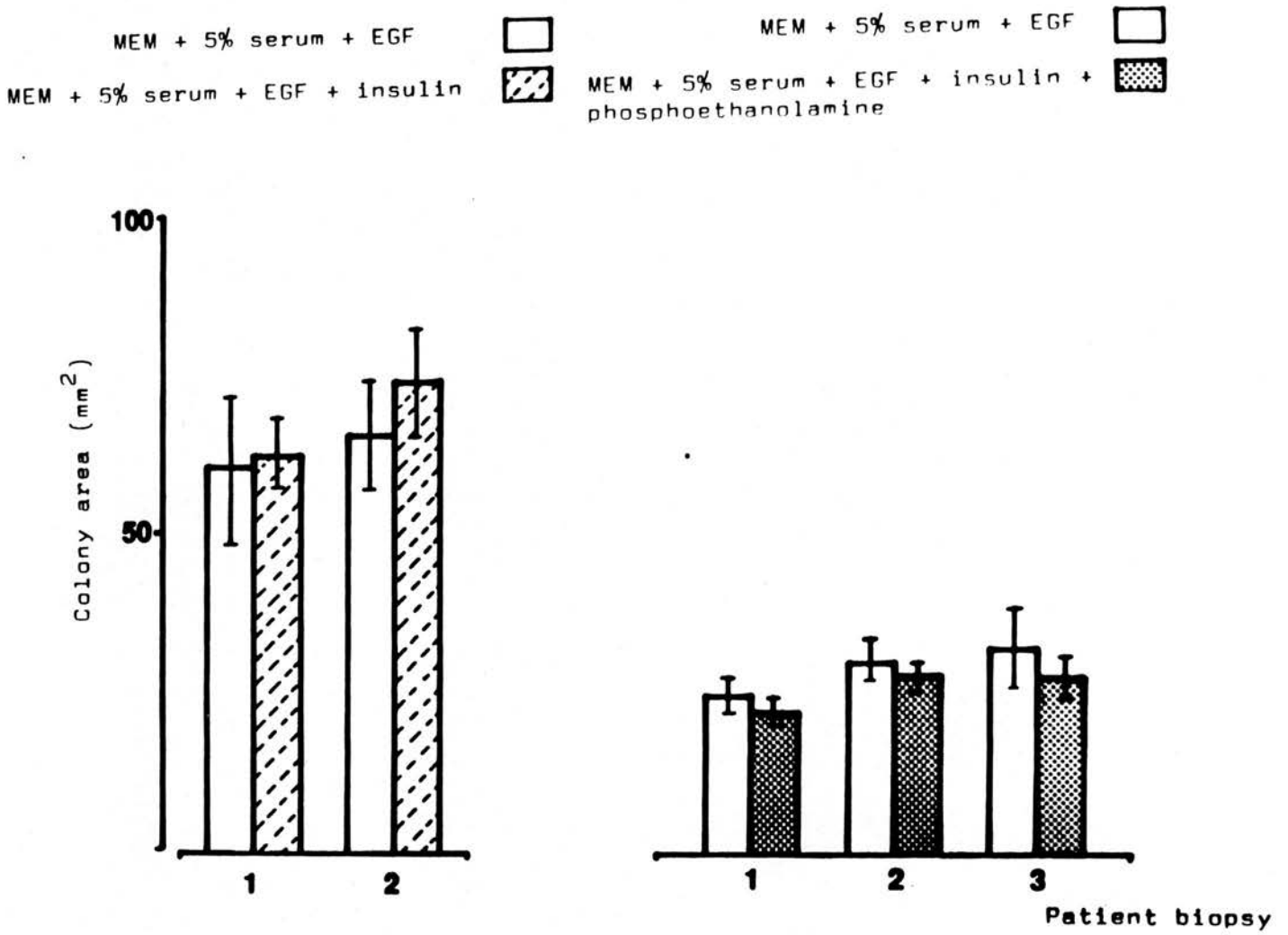


Fig.11 Areas of epithelial colonies (mm^2 ± S.E.) grown in various media.

TABLE 7

AREAS OF EPITHELIAL COLONIES (mm^2) GROWN IN MEDIUM CONTAINING 5% OR 10% SERUM WITH EGF AFTER 7 DAYS IN VITRO

Biopsy	Medium + 5% Serum + EGF	Medium + 10% Serum +EGF
1.	81.5 ±17.7	123.3 ± 18.1
2.	77.7 ± 8.0	235.2 ± 119.2
3.	129.1 ±60.5	91.4 ± 22.3

n=48

TABLE 8

AREAS OF EPITHELIAL COLONIES (mm^2) GROWN IN MEDIUM CONTAINING 5% SERUM AND EGF WITH AND WITHOUT INSULIN, AFTER 7 DAYS IN VITRO

Biopsy	Medium + 5% Serum + EGF	Medium + 5% Serum + EGF + IS
1.	60.1 + 13.5	62.3 + 5.4
2.	65.4 + 8.2	73.6 + 9.1

n=35

TABLE 9

AREAS OF EPITHELIAL COLONIES (mm^2) GROWN IN MEDIUM CONTAINING 5% SERUM AND EGF WITH AND WITHOUT INSULIN AND PHOSPHOETHANOLAMINE, AFTER 5 DAYS IN VITRO

Biopsy	Medium + 5% Serum + EGF	Medium + 5% Serum + EGF + IS + PEtn
1.	24.8 ± 2.9	19.9 ± 1.1
2.	29.5 ± 2.9	27.6 ± 2.2
3.	31.9 ± 6.7	26.9 ± 3.0

n=44

that growth was consistently slower without antibiotics than in antibiotic containing medium (Table 10, Fig. 12).

Good growth of oral keratinocytes was therefore achieved in the following medium:

Eagle's Minimum Essential Medium (x10 liquid)	10%
L-glutamine	2.0mM
Sodium pyruvate	1.0mM
Non-essential amino acids (x100)	1%
Sodium bicarbonate	10mM
Hepes buffer	20mM
Penicillin	$100Uml^{-1}$
Streptomycin	$100ugml^{-1}$
Fungizone	$2.5ugml^{-1}$
Epidermal Growth Factor	$10ngml^{-1}$
Human serum	5%

The point counting method used to assess the areas of culture dishes covered by epithelium and fibroblasts showed that the percentage area of culture dishes covered by fibroblasts remained very low in most cases with a mean value of 2.0% +/- 0.6%. Two of the twenty cultures grown in this medium showed larger areas of contamination by fibroblasts (10% and 14.9%) and perhaps a more accurate reflection of fibroblast contamination in these cultures is given by the median value of 0.5% (Table 11).

Attempts to subculture oral epithelium grown to near confluence in this medium were unsuccessful even in split ratios as low as 1:1.5.

TABLE 10

AREAS OF EPITHELIAL COLONIES (mm^2) GROWN IN MEDIUM WITH AND WITHOUT ANTIBIOTICS AFTER 7 DAYS IN VITRO

Biopsy	Medium With Antibiotics	Medium Without Antibiotics
1.	62.4 ± 1.6	38.8 ± 8.4
2.	84.5 ± 12.3	45.0 ± 5.5
3.	72.6 ± 10.9	50.2 ± 3.7

n=52

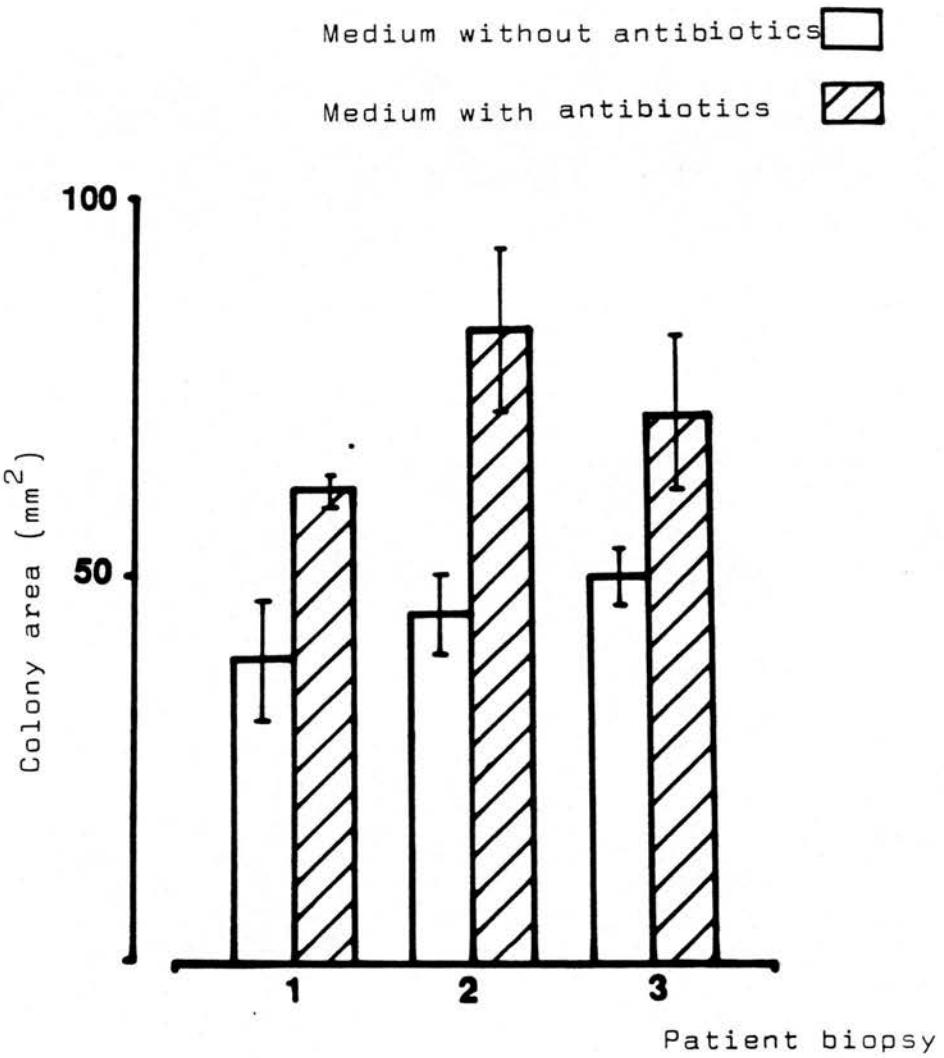


Fig.12 **Areas of epithelial colonies (mm^2 ±S.E.) grown in medium with and without antibiotics.**

TABLE 11

PERCENTAGE OF SURFACE AREA OF CULTURE OCCUPIED BY EPITHELIAL CELLS AND FIBROBLASTS AFTER 3-4 WEEKS IN VITRO ESTIMATED BY A POINT COUNTING METHOD (DUNNILL, 1968)

Culture No.	% Epithelium	% Fibroblasts	% Space	Points Counted
1	70.5	0.03	29.1	900
2	86.1	0.0	13.9	1188
3	82.8	0.0	17.2	1008
4	77.6	0.0	20.4	888
5	51.6	2.1	46.2	1044
6	72.1	0.0	27.8	1296
7	88.1	3.3	8.5	2092
8	72.2	10.3	17.6	2194
9	81.8	4.5	13.8	1947
10	88.4	0.05	10.0	1393
11	80.7	0.3	18.4	1658
12	82.2	0.3	17.2	3060
13	78.3	0.05	21.2	2197
14	91.0	0.5	8.5	2346
15	89.7	0.6	8.5	1348
16	56.8	14.9	28.3	2836
17	90.6	1.9	7.4	4326
18	83.6	0.0	16.3	1944
19	67.1	1.0	31.7	2690
20	55.6	1.0	43.4	1242
Mean	77.3 ± 2.6	2.0 ± 0.06	20.3 ± 2.6	1911

2.6.3.2 Growth of Keratinocytes from Oral Squamous Cell Carcinomas in Explant Culture

Eighteen (52%) of the oral squamous cell carcinomas produced epithelial outgrowths in explant culture. The lower success rate with the tumours stemmed mainly from the higher incidence of contamination (31%) in these cultures although some tumours simply failed to grow. Fibroblast contamination was also apparent in tumours cultured in this medium but again the rate of contamination estimated in 8 cultures by point counting remained low 1.4% +/- 1.1%.

Tumour cultures were more readily subcultured than normal gingival epithelium. In this study, three tumours which grew rapidly in primary culture were subcultured through 2-4 passages in a 1:2 split ratio.

Conclusion:

Successful explant cultures of oral keratinocytes were obtained in a selective medium. Having established that oral epithelial cells could be cultured *in vitro*, it was necessary to ascertain whether or not the characteristics of normal cells and tumour cells were retained *in vitro*.

2.7 CHARACTERISATION OF ORAL KERATINOCYTES GROWN IN VITRO

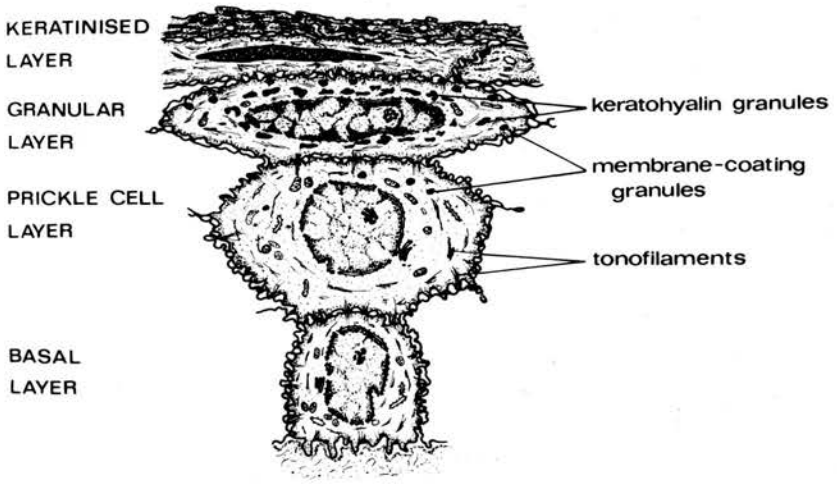
2.7.1 Introduction

In order to ensure that epithelial cells derived from non-neoplastic and neoplastic tissues cultured *in vitro* retained features associated with their tissue of origin, a number of parameters normally associated with normal and malignant cells were studied, including ultrastructure, thymidine labelling and karyotype.

Human epidermis and oral epithelium are composed mainly of keratinocytes in various stages of differentiation. Cells arising by division in the basal region of the epithelium undergo a process of differentiation to form a protective surface layer. Three types of epithelium are found in the oral cavity - ortho, para and non keratinized epithelia, each fulfilling different functions (Squier *et al* 1976). Despite the differences in the types of keratinization, similar patterns of differentiation occur in the deeper layers. Differentiation of orthokeratinized epithelium is shown schematically in Fig. 13. Cells in contact with the basement membrane overlying the lamina propria form the basal layer. These cells are primarily responsible for division and replacement of cells shed at the surface. Above the basal layer are several layers of isodiametric cells, which, because of the spiky appearance of their intercellular attachments, are referred to as the prickle cell layer or stratum spinosum. The basal and prickle cell layers together constitute approximately two thirds of the thickness of the

FIG. 13

Schematic diagram of skin layers.


KERATINISED LAYER
GRANULAR LAYER
PRICKLE CELL LAYER
BASAL LAYER
keratohyalin granules
membrane-coating granules
tonofilaments

epithelium and only in the remaining layers do clear patterns of differentiation emerge. During transition of cells from the basal layer, to the surface, epithelial cells continue to synthesize and accumulate cytokeratin tonofilaments which ultimately form the basis of resistance to mechanical injury. The prickle cell layer is succeeded by larger, much flatter cells in the stratum granulosum, which synthesize two types of granule: membrane coating granules (MCG) and keratohyalin granules (KHG). Membrane coating granules contain lipids and proteins which are extruded into the intercellular space and provide a permeability barrier which prevents unchecked diffusion in either direction. Within the stratum granulosum, the proteins which comprise keratohyalin granules contribute ultimately to the cornified envelopes which package the cytokeratin in the superficial layers of epithelium. These cells constitute the cornified layer of stratum corneum and contain dense eosinophilic deposits of keratin in an envelope of insoluble proteins. This pattern of differentiation is seen most clearly in orthokeratinized epithelium of the hard palate (Fig. 14). These cells form the protective surface and are shed in response to normal wear. Areas of the oral cavity less prone to mechanical forces form only parakeratinized epithelium. The surface cells retain pyknotic nuclei and a distinct granular layer is often difficult to recognise (Fig. 15). In non-keratinised epithelium it is much more difficult to distinguish clear cut zones. Cells in the outer layer retain apparently normal nuclei and show little or no eosinophilia (Fig. 16).

FIG. 14

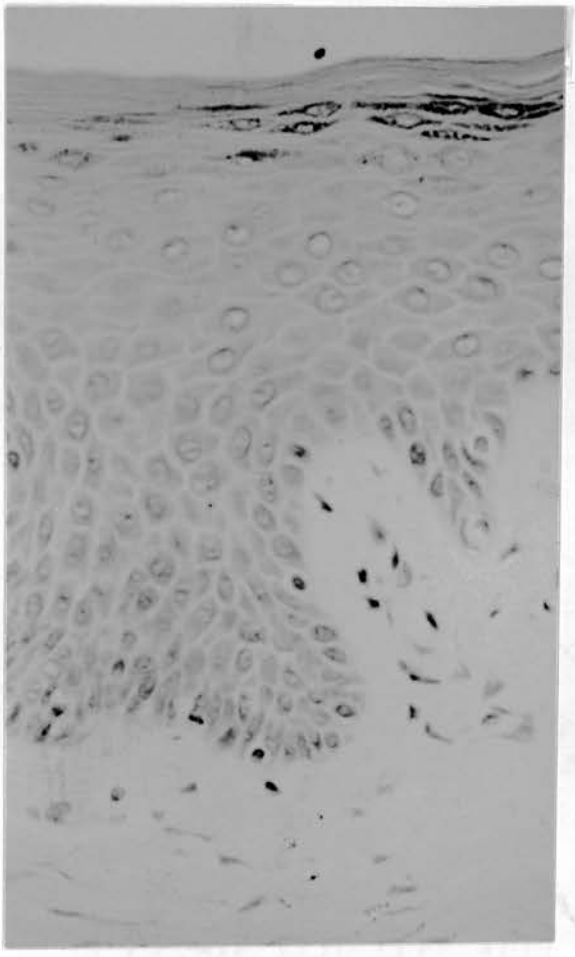
Orthokeratinised epithelium from the hard palate. Prominent granular area and keratinised layers are apparent. Stained with haematoxylin and eosin (x300 mag).

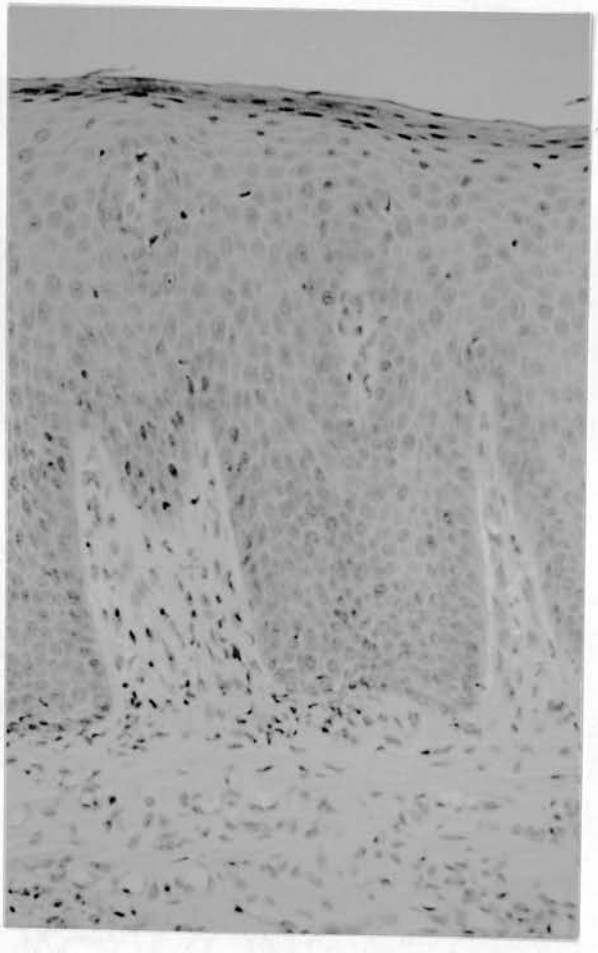
FIG. 15

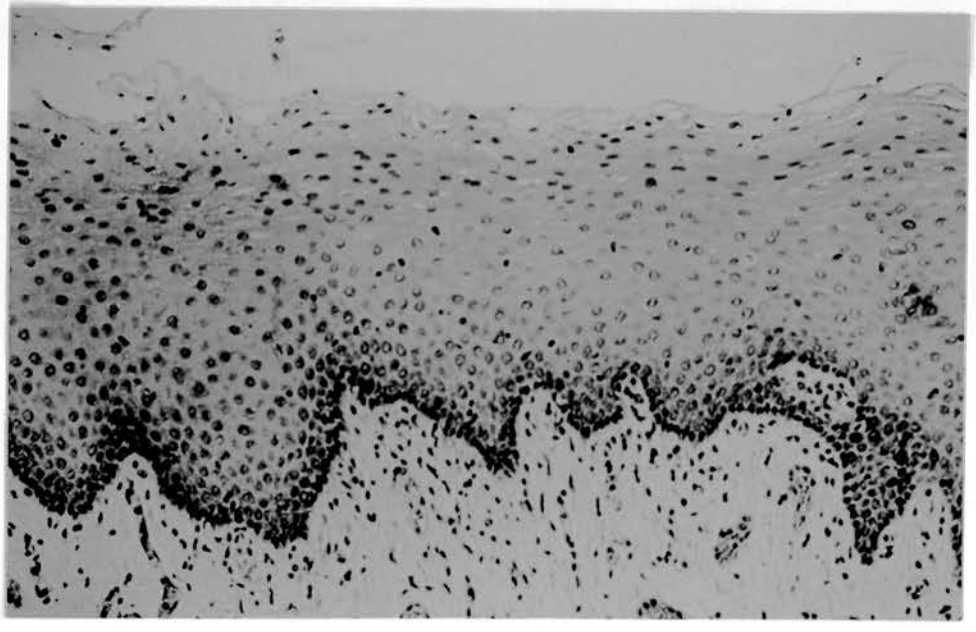
Parakeratinised epithelium. A granular layer is not apparent and surface cells contain pyknotic nuclei. Stained with haematoxylin and eosin (x150 mag).

FIG. 16

Non keratinised epithelium from cheek. A granular layer is not present and surface cells retain apparently normal nuclei. The epithelium has a less distinct ridged pattern. Stained with haematoxylin and eosin (x150 mag







Differentiation, occurs as cells move from the basal layer to the cornified layer, and involves other changes which are only evident in the electron microscope.

Electron micrographs of a gingival biopsy demonstrate that the basal cells (Fig. 17) are columnar, electron lucent and relatively undifferentiated. The cells do, however, contain organelles associated with the basic synthetic apparatus of the cell (Fig. 18), ribosomes, rough endoplasmic reticulum and mitochondria being present. Few cytokeratin tonofilaments are present except at sites of desmosomal attachment between cells. Within the basal layer are small elongated darkly staining cells (Fig. 19) with a very low nuclear to cytoplasmic volume. These cells have been noted by others (Klein-Szanto and Slaga, 1981) and these may be artefacts as a result of fixation methods.

The fundamental characteristic of all keratinocytes is the synthesis of cytokeratin tonofilaments. These intracellular protein strands arrange together to form tonofibrils. The tonofilaments are retained with the cell and are organised into a three dimensional network linked to desmosomes, which together distribute mechanical stresses evenly throughout the epithelium (Fig. 20). Desmosomes, the commonest intercellular junctions, form as a result of structural modifications of the plasma membrane and occupy areas of adjacent plasma membranes. Intracellular thickening can be seen forming attachment plaques, into which the tonofilaments are inserted (Fig. 21). Desmosomal junctions

FIG. 17

Gingival biopsy. Section through basal layer and connective tissue (C). Columnar cells are interspersed with "dark cells" (x3500 mag).

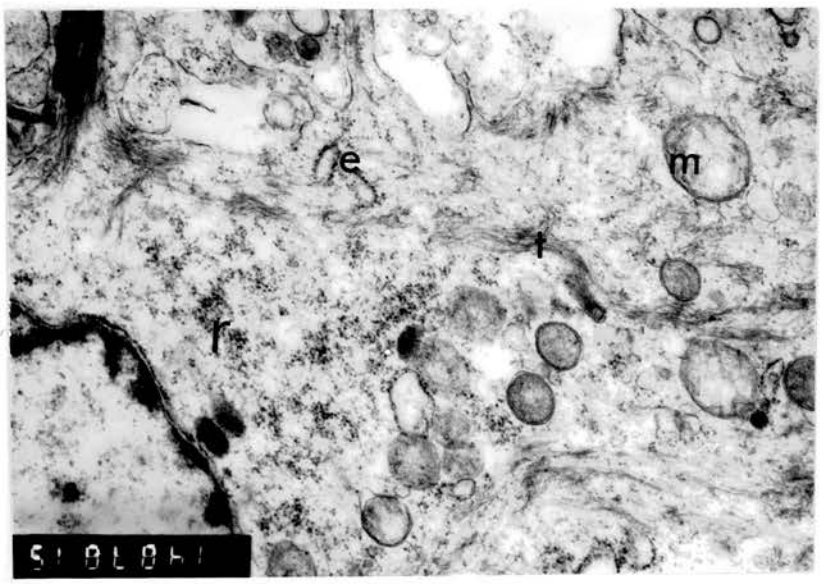
FIG. 18

Cytoplasm of basal cells containing ribosomes (R), rough endoplasmic reticulum (E), mitochondria (M) and few cytokeratin tonofilaments (T) (x14,000 mag).

FIG. 19

Gingival biopsy. "Dark" cells within basal layer (x14,000 mag).


C


e
m
r

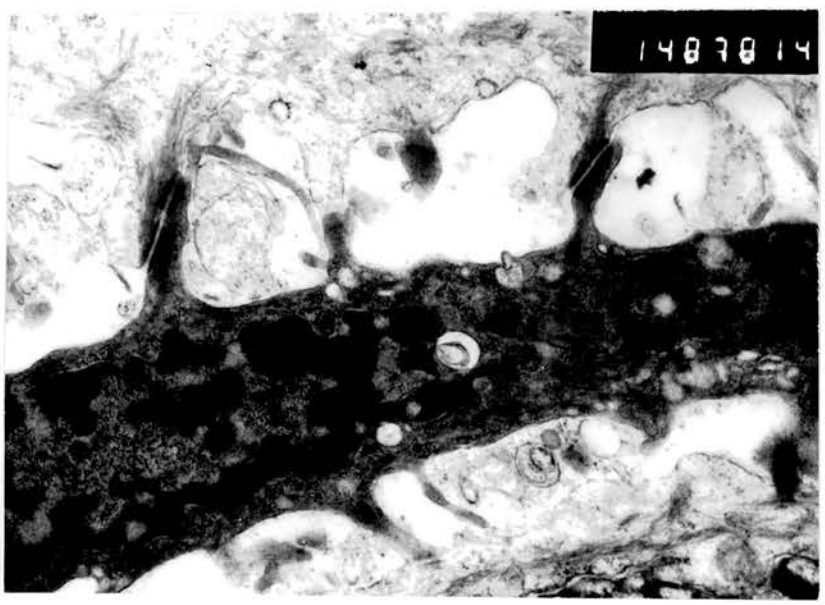
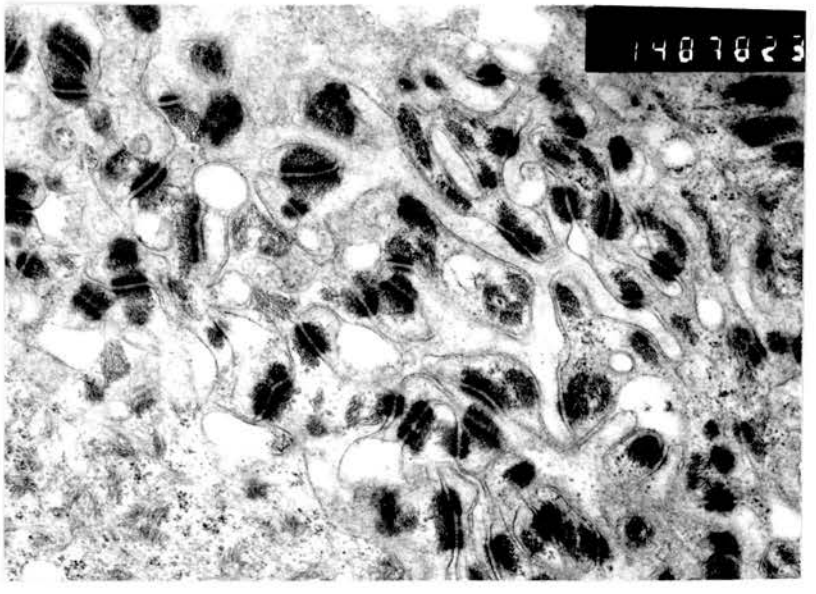


FIG. 20

Gingival biopsy. Section through desmosomes where several cells connect in the prickle cell layer (x14,000 mag).

FIG. 21

Gingival biopsy. Desmosomes between two prickle cells. Intracellular thickening can be seen forming attachment plaques into which the tonofilaments are inserted. Each desmosome shares the cytoplasm of two cells (x35,000 mag).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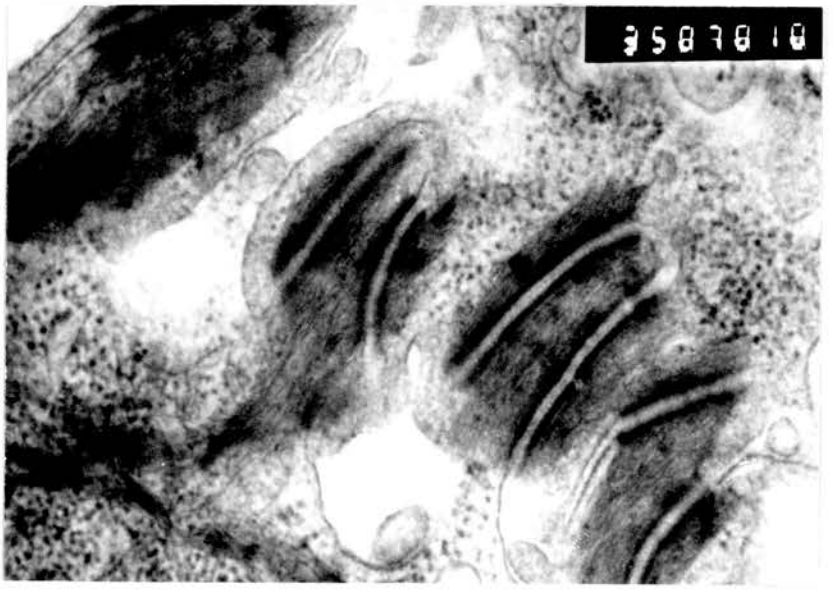
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are entirely specific for epithelium.

Changes accompanying differentiation in oral epithelia are an increase in cell size, as cells move out of the basal layer into the prickle cell layer, and the synthesis of additional structural proteins (Figs. 22 and 23). In gingival epithelium, a granular layer, in which membrane coating granules and keratohyalin granules would normally be synthesized, is often difficult to recognise or may even be absent. However, features associated with differentiation such as the loss of subcellular organelles and the accumulation of cytokeratin are usually seen in the upper layers of cells (Fig. 24).

Electron microscopic examination of tumour cells from biopsy specimens has shown that a number of alterations in the subcellular structure of these cells are consistently associated with a malignant phenotype. Although no single change is pathognomonic, the spectrum of changes within tumour cells can be a reliable indicator of malignancy (Ghadially, 1975). Such changes include enlargement and marked invagination of nuclei (Ghadially, 1975), and intranuclear inclusions are much more frequent in tumour cells (Ghadially and Perry, 1966) although the majority of these are in fact pseudoinclusions, which because of invagination of the nuclear membrane, appear to lie within the nucleus. Chromatin granules appear in a variety of tumour cells and may be indicative of aberrant protein synthesis since these granules have been found after administration of certain drugs (Meine et al 1971; Daskai

FIG. 22

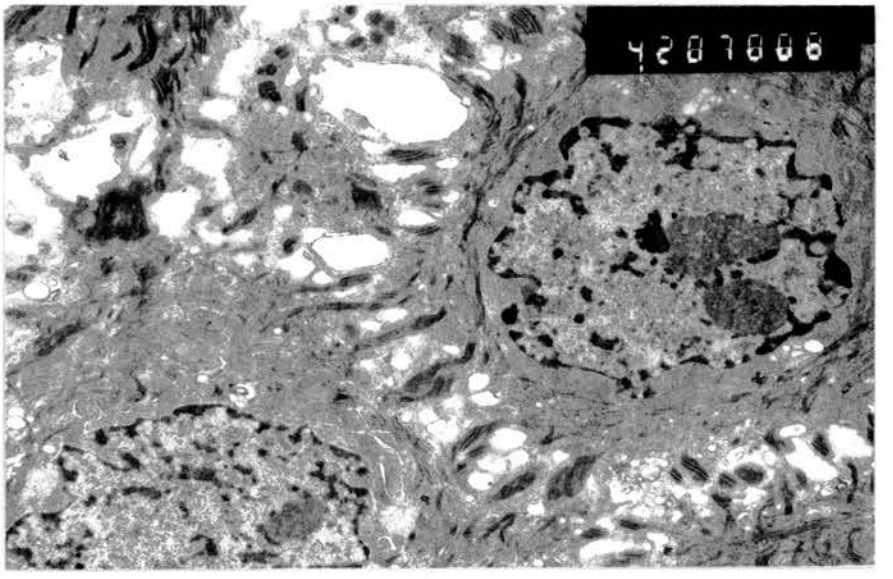
Gingival biopsy. Prickle cell layer. Increased synthetic activity in the stratum spinosum results in the production of cytokeratin tonofilaments, which group together to form tonofilament bundles (x4,200 mag).

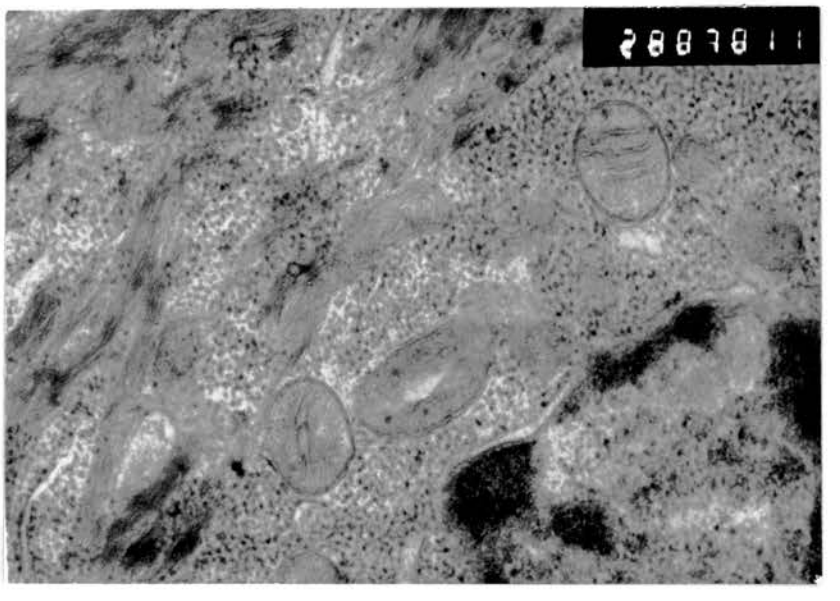
FIG. 23

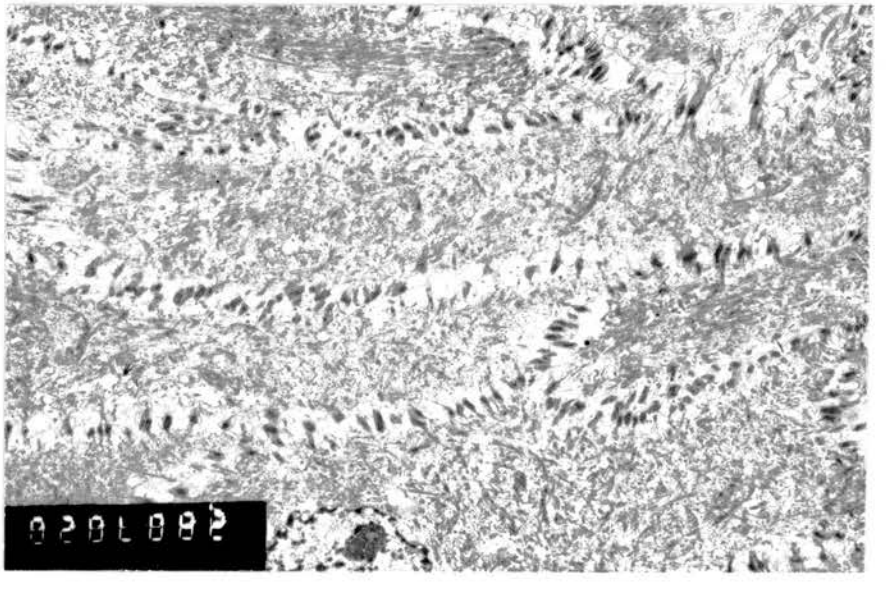
Gingival biopsy. Cytoplasm of a cell in the prickle cell layer. Increased density of tonofilaments, ribosomes and mitochondria are apparent (x28,000 mag).

FIG. 24

Gingival biopsy. No distinct granular layer is present, however, surface cells are characterised by dense cytoplasmic tonofilaments and a loss of subcellular organelles. Cells at surface of section contain no nuclei (x2,800 mag).







et al 1975; Norton et al 1975). Other subcellular changes in tumour cells include a reduction in the number of mitochondria (Lehninger 1965; Fawcett, 1967) as a result of glycolytic metabolism in the cells. Furthermore, these mitochondria often have a pleomorphic appearance and are often biochemically defective (Sordahl et al 1969; Pederson, 1970).

Increased growth rate of tumours is also a consistent finding in tumours in vivo (Kerr et al 1983; Hobson and Denekamp, 1984). The proliferation rate of normal oral keratinocytes and keratinocytes derived from malignant tissue was also measured in cultured cells by determining the thymidine labelling index (TLI).

Changes in chromosomal constitution are also indicative of malignant cells. In vitro only one spontaneously transformed human keratinocyte cell line (HaCaT) has been reported (Fusenig et al 1988). This cell line, derived from normal human adult skin (back), is aneuploid with 3 distinct chromosome markers. Despite the rarity of this event, spontaneous chromosomal abberations do occur during the culture of normal human keratinocytes (Peters et al 1988). Using foreskin keratinocytes these authors reported that very early cultures (from 2 days) exhibit 5 - 10% tetraploid metaphases and that this number increases steadily during the first month of culture up to 25%. As cells senesce, however, the majority of cells in culture are again diploid. Chromosomal abberations may therefore be more common than previously thought in primary cultures of keratinocytes.

2.7.2 Materials and Methods

2.7.2.1 Electron Microscopy of Cultured Oral Keratinocytes

Epithelial explants were grown on Lux permanox culture dishes (60 mm diameter) which are compatible with EM solvents. Cells were fixed *in situ* in ice cold 4% buffered gluteraldehyde (TAAB) in 0.1M sodium cacodylate buffer containing 0.05M calcium chloride and 0.02M hydrochloric acid (BDH Chemicals). After 24 hours in fixative at 4°C the cells were rinsed twice in 0.1M sodium cacodylate buffer and post fixed in 1% osmium tetroxide (BDH Chemicals) in 0.1M sodium cacodylate buffer for 1h. Tissues were dehydrated through a series of alcohols to absolute ethanol. After three changes in absolute ethanol for 30 min., tissues were placed in two changes of epoxypropane (Agar Scientific EM grade) for 30 min. and then placed in a 50:50 mixture of epoxypropane: araldite (Agar Scientific) for a minimum of 3h. Tissues were left overnight at room temperature in fresh epoxypropane:araldite mixture. The following day tissues were placed in fresh araldite and placed in a 60°C oven for two days. Small blocks of tissue (approximately 1 mm^3) were mounted on dowling rod with sealing wax before sectioning in an LKB ultramicrotome.

Thin sections (gold to silver) were mounted on copper grids (E200) and stained in 0.2% lead citrate in 0.1N NaOH for 2 min. followed by washing in distilled water. Grids were then stained with a saturated solution of uranyl acetate in 50% alcohol for 10-15 min. in the dark, and rinsed in 50%

alcohol before viewing in a Joel 100S electron microscope.

2.7.2.2 Cytokeratin Staining

Oral keratinocytes cultured *in vitro* were stained using a conventional PAP method which is described fully in section 4.2.1 in Chapter 4. In this procedure a polyclonal antiserum to cytokeratins (5+14), raised in rabbits (Orthoimmune), was used at a dilution of 1:400 in the initial staining stage (reviewed by Moll *et al* 1982).

2.7.2.3 Autoradiography

Epithelial cultures were pulse labelled with tritiated thymidine, 20uCi per culture dish, (sp. act 40-60 $CimMol^{-1}$; Amersham International) for 1h. Control cultures contained no tritiated thymidine. Labelling was stopped by washing the cultures in ice cold PBSA and fixing in 10% neutral buffered formalin. In a darkroom, K5 nuclear emulsion (Ilford) was melted at 50°C and then diluted 2:1 in 2% glycerol. Epithelial cells in culture dishes were coated with an even film of emulsion, allowed to dry and placed in light tight boxes. After exposure for one week at 4° C, the films were developed in D19 developer (Kodak) for 3.5 min. and fixed in Unifix (Kodak) for 9 min at room temperature. The culture dishes were washed and tissue stained in haematoxylin and eosin. The number of labelled cells in each culture was determined from counts of no less than 750 cells in two discrete outgrowths from each patient culture. The labelled

cells counted (6 grains per nucleus) were expressed as a percentage of the total number of cells counted to give the thymidine labelling index (TLI).

2.7.2.4 Karyotyping

Log phase cultures of oral keratinocytes were treated with 100 $ugml^{-1}$ colchicine (BDH Chemicals) for 2h at 37°C. The medium was then removed and the cultures trypsinised in the normal way. The cell suspension was centrifuged at 200 g for 5 min. and the cell pellet gently resuspended in 0.075M potassium chloride for 4 min. at room temperature. The cells were centrifuged at 200g for 5 min. and the supernatant aspirated except for one drop in which the cell pellet was resuspended. Ice cold methanol: acetic acid (3/1 vol:vol) was added dropwise with constant agitation up to a total volume of 3 ml and the suspension left on ice for at least 30 min. The suspension was then centrifuged at 200g for 5 min. and 3 ml of fresh fixative added. The cells were washed 3 more times in fixative and, after the final wash, the pellet was resuspended in 0.5 ml of fixative. Drop preparations were made onto pre-cleaned wet microscope slides from a height of 30 cm. Slides were air dried and stained for 5 min. in Giemsa stain (BDH Chemicals) and mounted in buffered glycerol.

From peparations of each of the four cultures of normal gingival epithelium the first five intact metaphase spreads were selected and photographed. Photographic enlargements were made and the chromosomes counted.

2.7.3 Results

2.7.3.1 Light and Electron Microscopy of Cultured Gingival Epithelium

Vertical sections through two week old cultured gingival epithelium showed a structure which is 2-4 layers thick (Fig 25). A distinct population of relatively uniform basal cells can be seen on the under surface of the culture. As the cells moved out of the basal layer they enlarged and flattened, assuming an appearance reminiscent of prickle cells *in vivo*. Cells on the surface of the culture retained their nuclei a feature of cells in non-keratinised epithelium *in vivo*.

After 1-4 days in culture, phase contrast micrographs showed polyclonal cells migrating from part or the whole of the circumference of the explanted tissue (Fig. 26). Cells continued to divide for up to six weeks producing a continuous sheet of epithelium with intercellular bridges between the epithelial cells which can be clearly identified (Fig. 27). Very occasionally small circular areas of the outgrowth remained devoid of cells (Fig. 28). These may have resulted from contaminating endothelial cells co-migrating from the explant with the epithelial cells and attempting to form vessels within the outgrowth. Epithelial cells rarely grew into these spaces.

Cells frequently migrated from the edge of the epithelial outgrowths but maintained contact with other

FIG. 25

Vertical section through normal cultured oral epithelium after four weeks *in vitro*. Basal cells (B) attached to the surface of the dish are columnar in shape. Suprabasal cells retain their nuclei. Stained with haematoxylin and eosin (x400 mag).

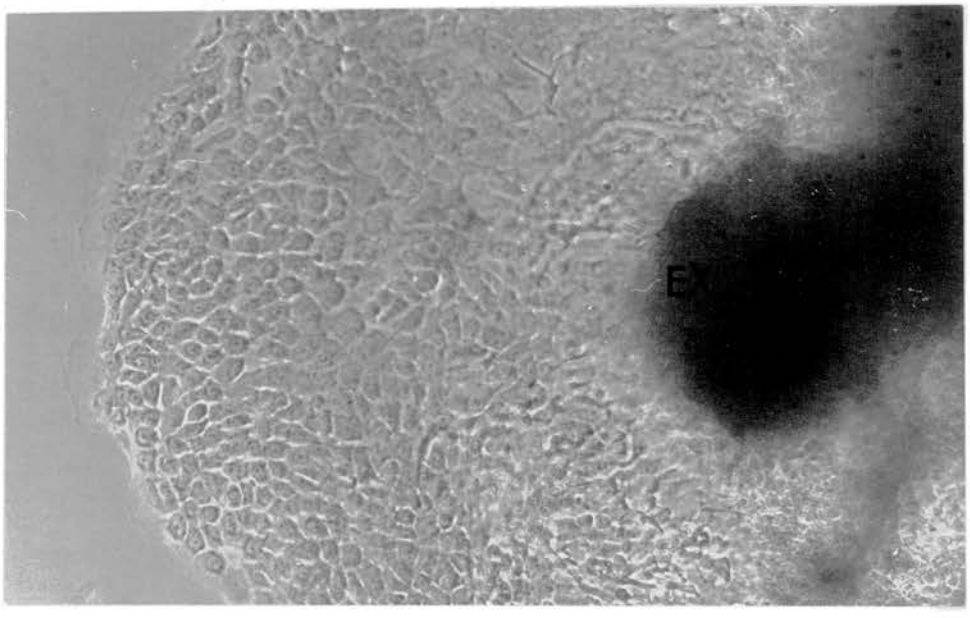
FIG. 26

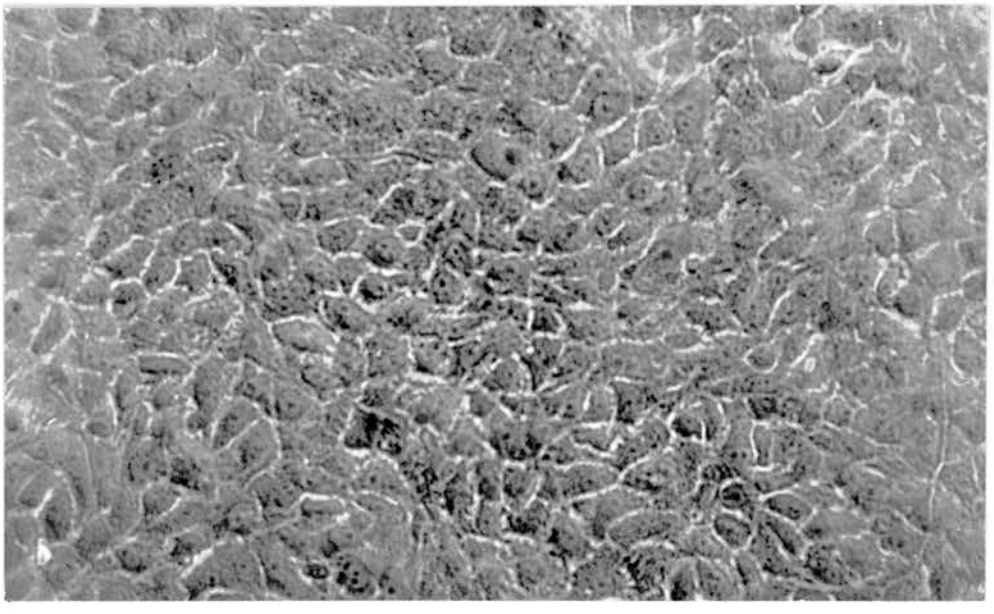
Oral epithelial cells in culture migrating from explanted tissue (Ex) (x100 mag).

FIG. 27

Confluent sheet of oral keratinocytes after 4 weeks *in vitro* (x200 mag).


B


E



epithelial cells by filopodia (Fig. 29). Microspikes and lamellopodia, which are smaller than filopodia (Fig. 30) were also a feature of the lateral edges of marginal epithelial cells. All such pseudopodia are involved in movement of epithelial cells, the fluidity and shape of which vary with the time at which they are photographed.

In the electron microscope, vertical sections through 2-3 week old outgrowths (Fig. 31) showed a similar pattern of growth to that seen in light micrographs. In these sections features of differentiation of cells were easier to recognise. Cells attached to the culture dish contained nuclei and had a larger cytoplasmic volume than in cells which had moved towards the surface of the culture. Cells nearer the surface became elongated and accumulated cytokeratin filaments. Occasional lipid droplets were found in these cells.

Sections of this nature were extremely difficult to cut because of their position at the surface of the araldite blocks and because of their thinness. Occasionally, in sections which had been cut more obliquely, keratinocytes could be seen sloughing from the surface of the culture (Fig. 32). The detachment sites were characterised by numerous microvilli. Such cells also contained a dense cytokeratin complement, although the remains of organelles were sometimes seen including keratohyalin granules.

Tangential sections through cultured cells showed that keratinocytes possessed well rounded nuclei containing mostly

FIG. 28

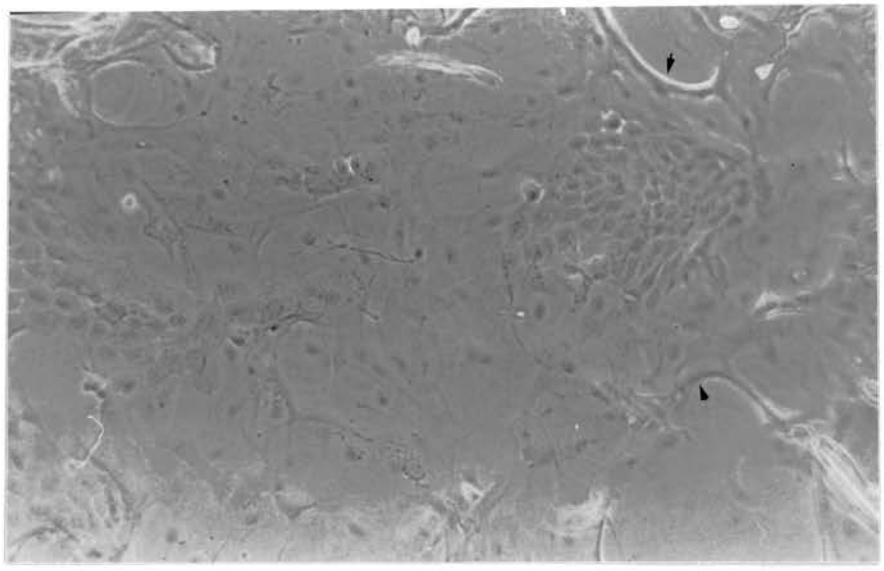
Oral keratinocytes *in vitro*, showing spaces possibly bound by endothelial cells (x100 mag).

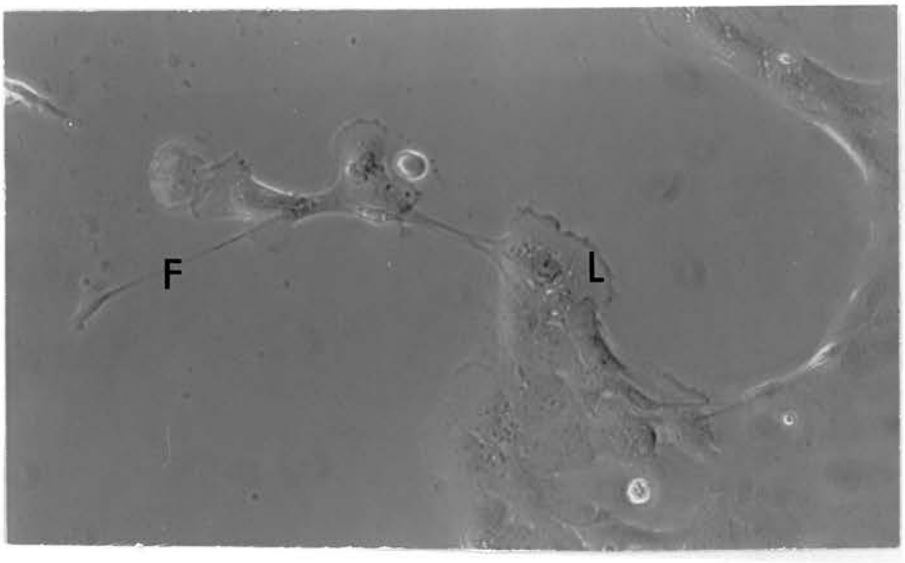
FIG. 29

Oral keratinocytes *in vitro*. Cells migrating at the edge of an epithelial colony producing filopodia (F) and flattened lamellopodia (L) or "ruffles" (x200 mag).

FIG. 30

Oral keratinocytes *in vitro*, showing dense keratinisation at the leading edge of 4 week old cultures (x100 mag).




F
L

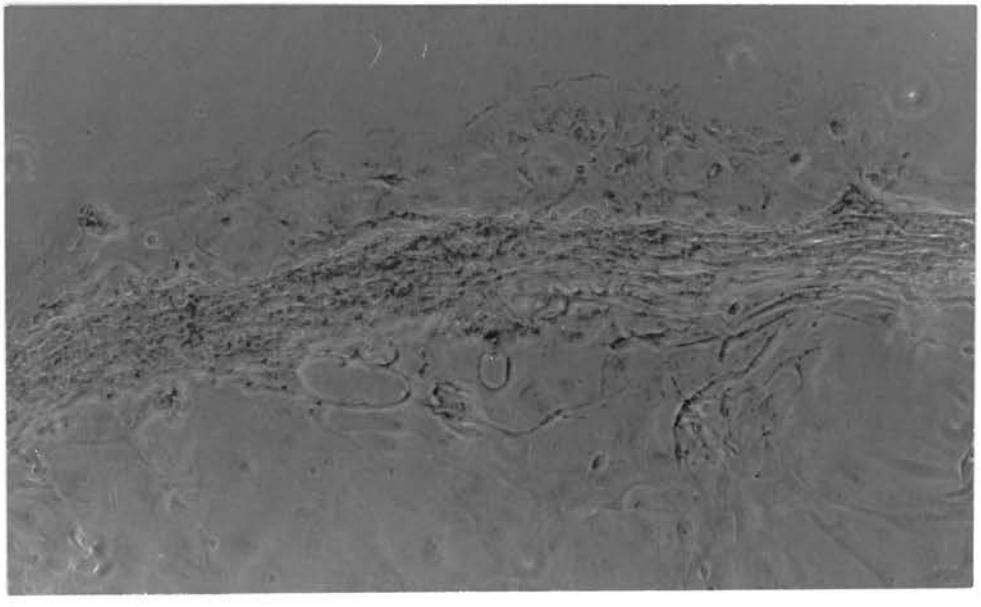
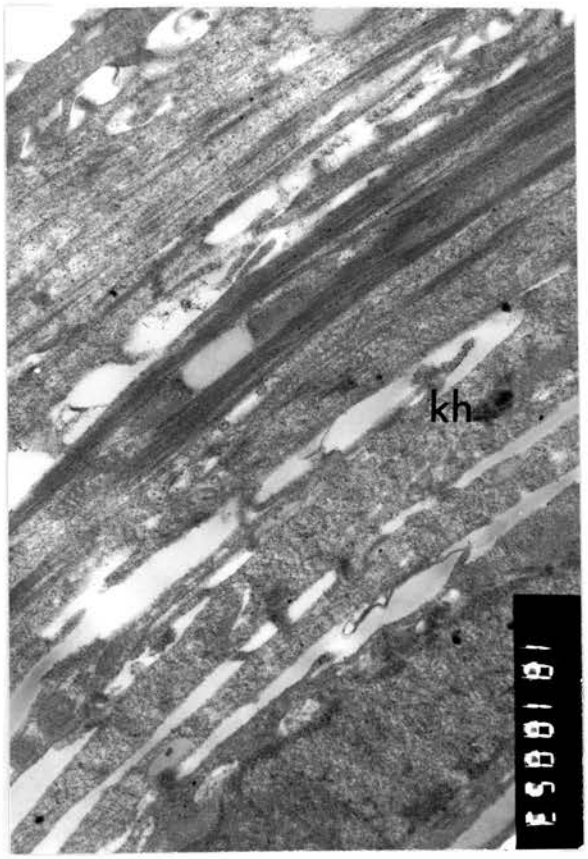


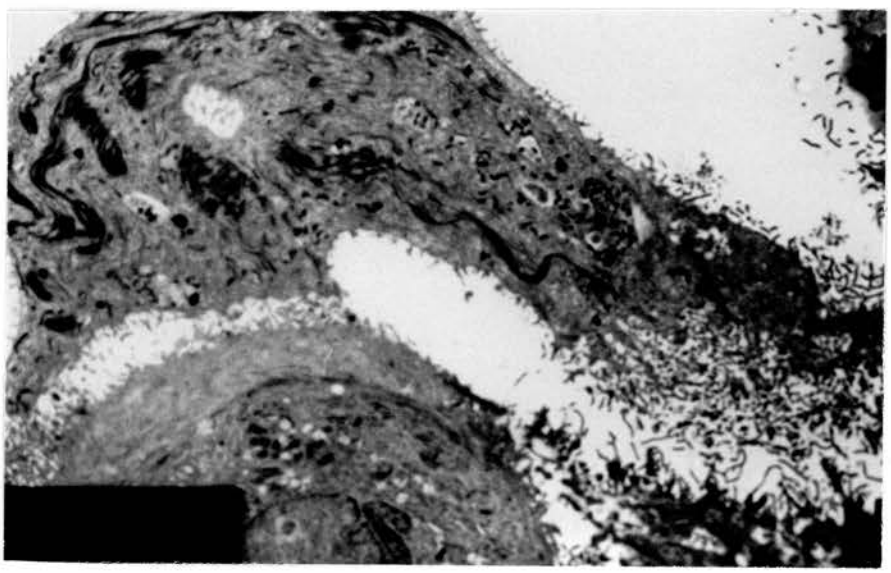
FIG. 31

Electron micrograph through cultured oral keratinocytes. Nucleated cells are present in the basal layer. Suprabasal cells are elongated, containing keratohyalin granules (kh) and surface cells contain dense cytokeratin tonofilaments (x18,000 mag).

FIG. 32

Keratinocyte sloughing from the surface of the cultured cells. The detachment of keratinocytes is characterised by numerous microvilli (x18,000 mag).


kh
ES8801 81



euchromatin and one or two nucleoli (Fig. 33). The cytoplasm of the cells contained cytokeratin, tonofilaments, numerous free ribosomes, mitochondria, occasional vacuoles and lipid inclusions. No Golgi bodies were identified in any of the EM sections of cultured cells.

Desmosomes were identified at all levels in the tissue but were more numerous in the intermediate cell layers (Fig. 34). In the superficial layers desmosomes were sparse. Keratohyalin granules were sometimes observed in superficial cells (Fig. 35).

Many mitoses were observed in cultured cells (Fig. 36). It was possibly due to the high mitotic activity, leading to a rapid transition of cells from the basal to superficial surface of the culture, that insufficient time was available to cells to synthesise membrane coating granules. These were not found in cultured cells.

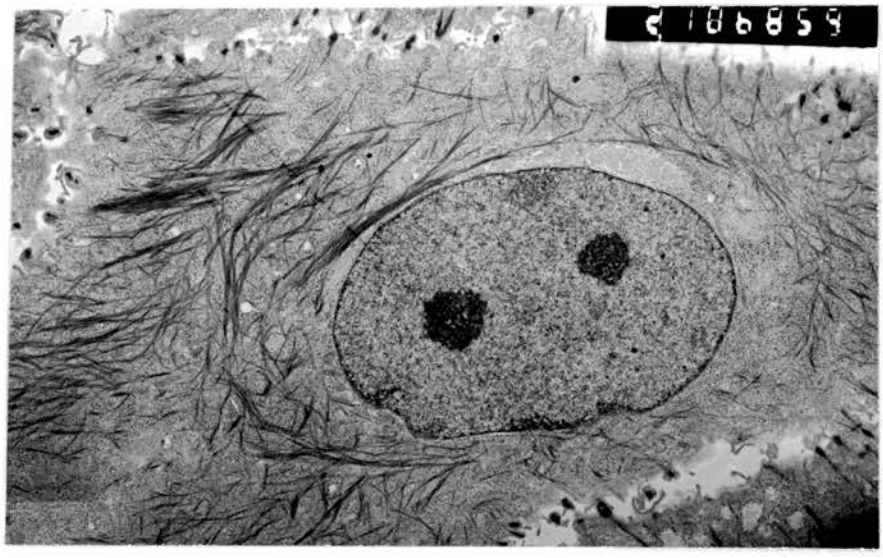
Cells other than keratinocytes may also persist in sections of cultured keratinocytes; with macrophages, mast cells and white blood cells being identified in epithelial cultures (Figs. 37 and 38). These cells were probably contaminants from explanted tissues. It is not known whether these cells have the potential to divide under the conditions pertaining, but on the basis of cell counts from randomly selected EM grids they constituted less than 3% of the cultured cell population (Table 12). Other cells such as melanocytes, Langerhans cells and Merkel cells, which are normally present in oral epithelium were not identified. This may reflect their inability to replicate under the

FIG. 33

Tangential section through oral keratinocyte *in vitro* (x2,100 mag).

FIG. 34

Desmosomal junctions between cultured oral keratinocytes *in vitro* (x18,000 mag).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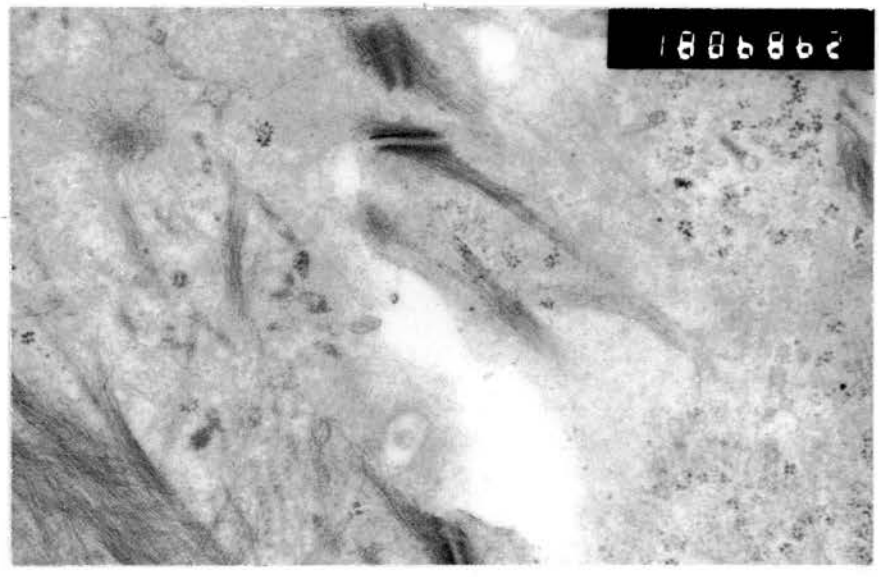
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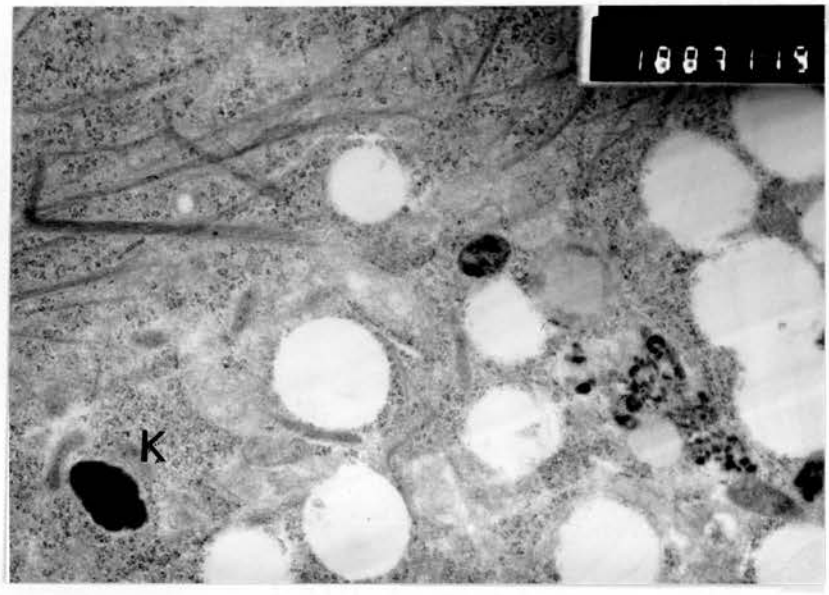
FIG. 35

Keratohyalin granules (K) in the more superficial layer in keratinocyte cultures (x18,000 mag).

FIG. 36

Cells in mitosis in keratinocyte cultures (x5,600 mag).


K

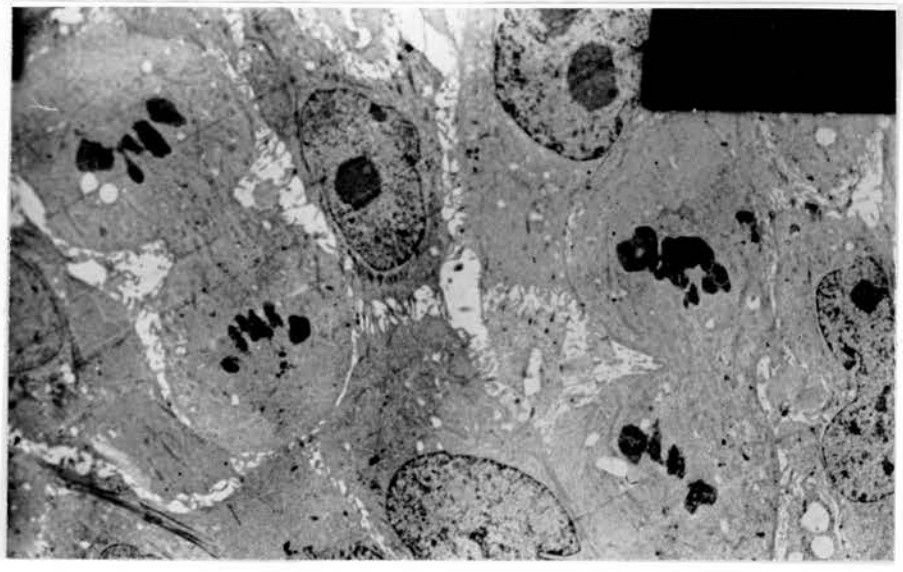
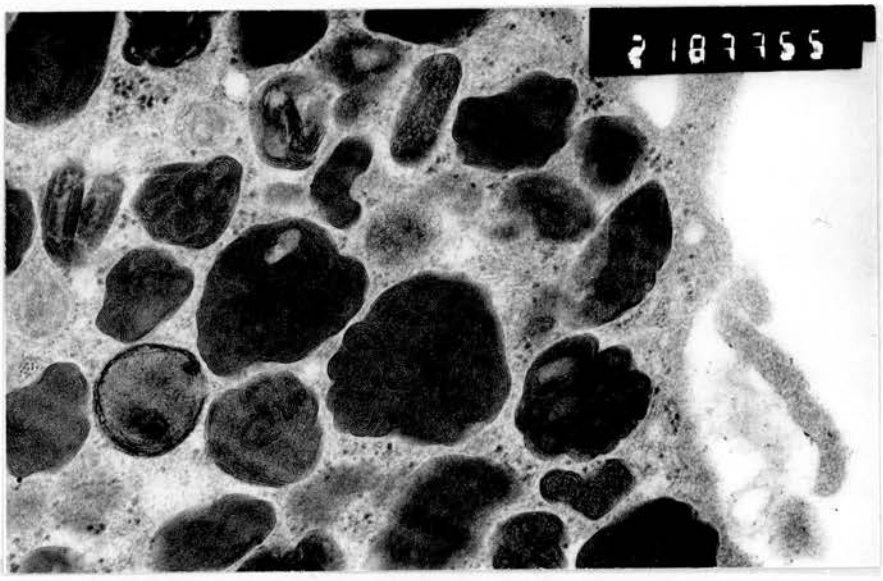


FIG. 37

Mast cell within keratinocyte culture after 4 weeks *in vitro*. Granules display a predominantly scrolled pattern (x21,000 mag).

FIG. 38

Macrophage within keratinocyte culture after 2 weeks. Macrophage is bound by keratinocytes which display prominent cytokeratin tonofilaments (x4,200 mag).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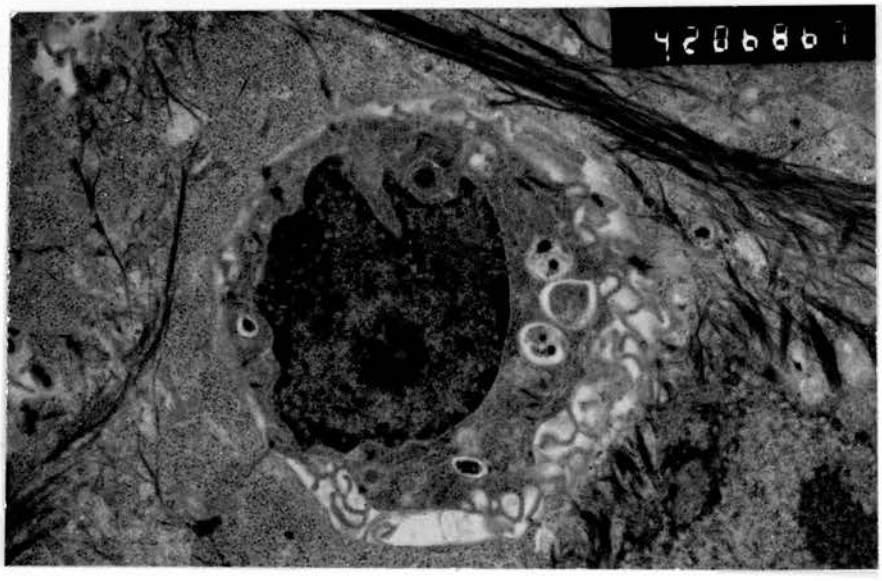
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TABLE 12

IDENTIFICATION OF CELL TYPES IN EM SECTIONS OF CULTURED GINGIVAL KERATINOCYTES

Cell Types	Keratinocytes %	White Blood Cells %	Macrophages %	Mast Cells %	Fibroblasts %	Unidentified %
Biopsy						
1.	86.1	0	6.3	0	0	7.6
2.	83.9	2.1	0.7	2.2	0	11.1
3.	99.1	0	0	0	0	0.9
4.	99.2	0	0	0	0	0.8
Mean	92.1	0.5	1.8	0.6	0	5.1

n=502

culture conditions provided. Fibroblasts were known to be a minor contaminant in cell cultures but were not identified in the EM sections. Cusp shaped and circular cells were observed in phase contrast micrographs of cultures of oral keratinocytes. These were thought to be endothelial cells arising from capillaries in connective tissue. No Weibel-Palade bodies were observed in cultured cells, however, these are often absent in cultured capillary endothelial cells (Gimbrone et al 1979). A proportion of the cells could not be identified simply because of the plane of section.

2.7.3.2 Light and Electron Microscopy of Cultured Oral Squamous Cell Carcinomas

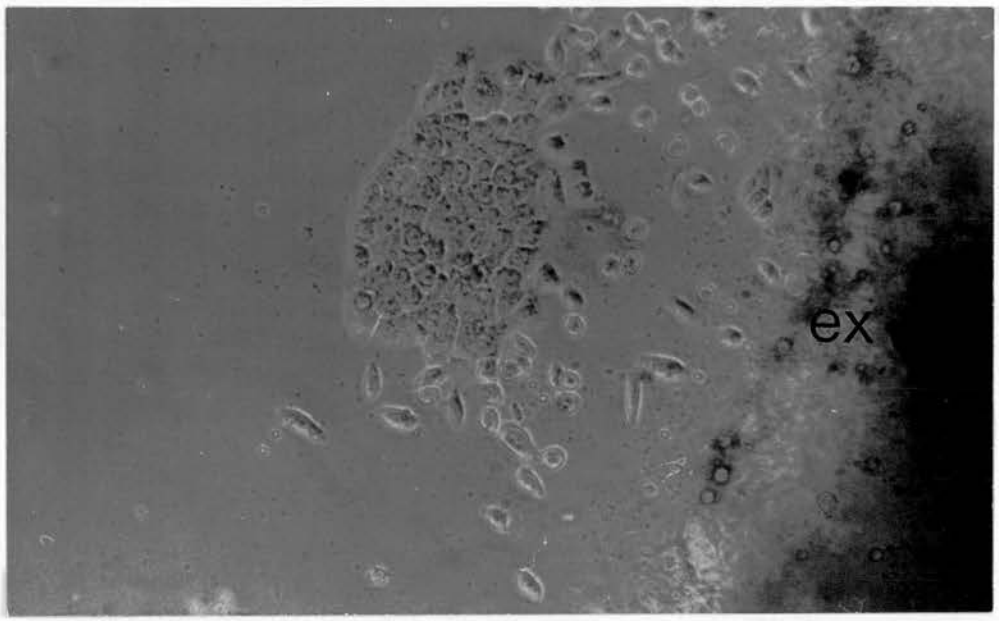
In successfully cultured tumours, epithelial cells grew from the explanted tissue after 1-7 days in vitro but, more usually, were apparent after 1-2 days (Fig. 39). Unlike gingival epithelium, tumour epithelium rarely grew as a continuous sheet (Fig. 40). The loss of adhesion between tumour cells resulted in islands of epithelium appearing at some distance from the explant site. The islands of cells continued to grow for up to six weeks and in many cases formed confluent layers of cells (Fig. 41). Subcultures of tumour epithelium through 2 - 4 passages had a similar phenotype to the parent culture (Fig. 42). Primary and passaged tumour cultures retained an epithelial appearance with distinctive intercellular bridges between cells. Tumour cells migrated in a similar manner to normal oral

FIG. 39

Keratinocytes migrating from the edge of explanted tumour tissue (ex) after 2 days *in vitro*, showing the loss of adhesion between cells (x100 mag).

Fig. 40

Islands of keratinocytes growing from explanted tumour tissue after 7 days *in vitro*. Cells retain the typical cuboidal appearance of epithelial cells but fail to grow in a continuous sheet (x200 mag).


ex

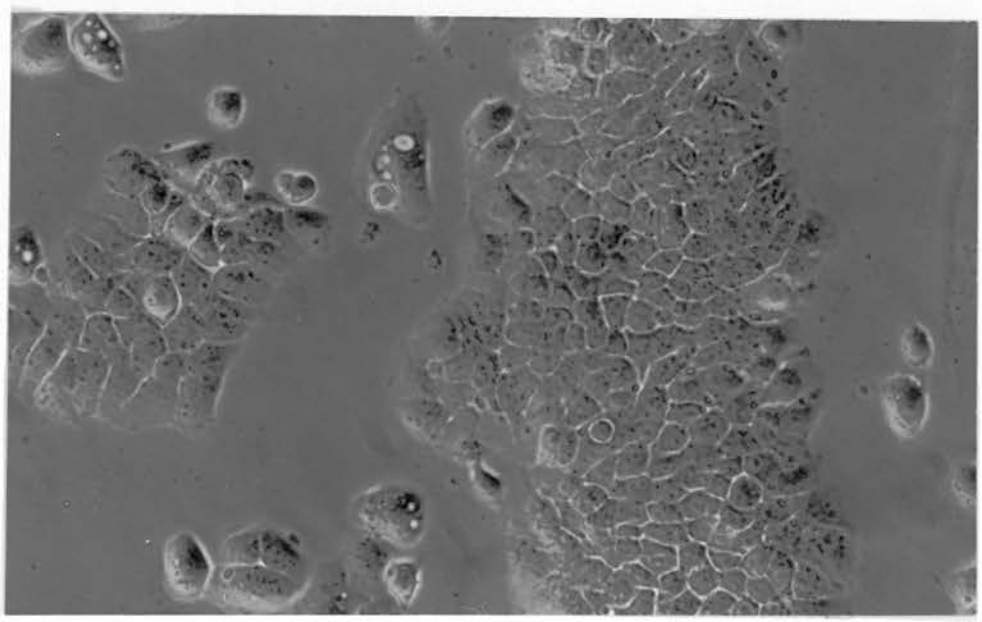


FIG. 41

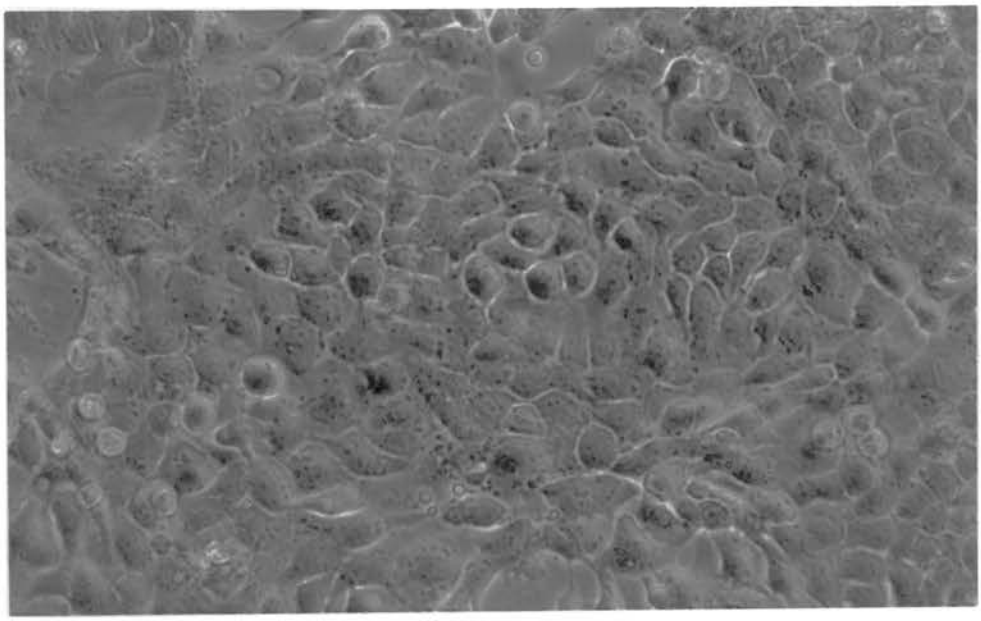
Almost confluent culture of oral tumour keratinocytes after two weeks *in vitro* (x400 mag).

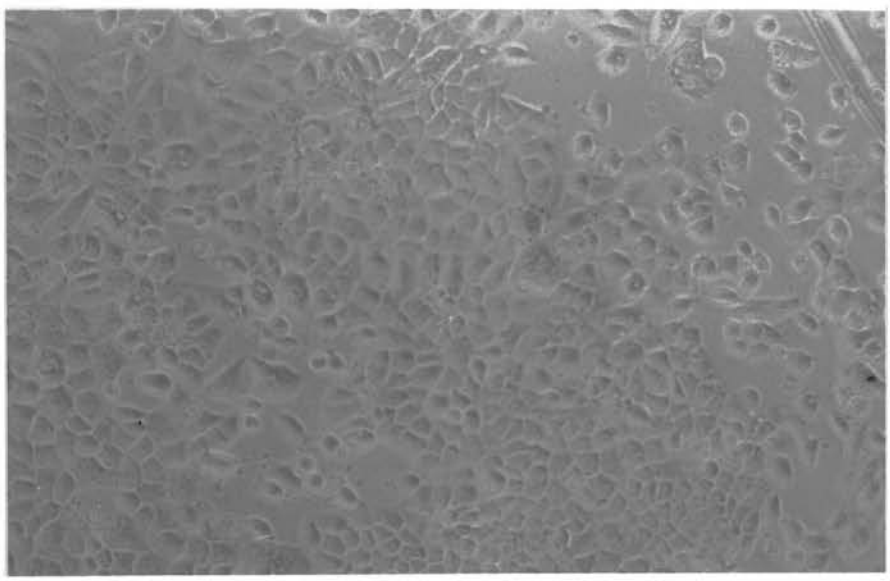
FIG. 42

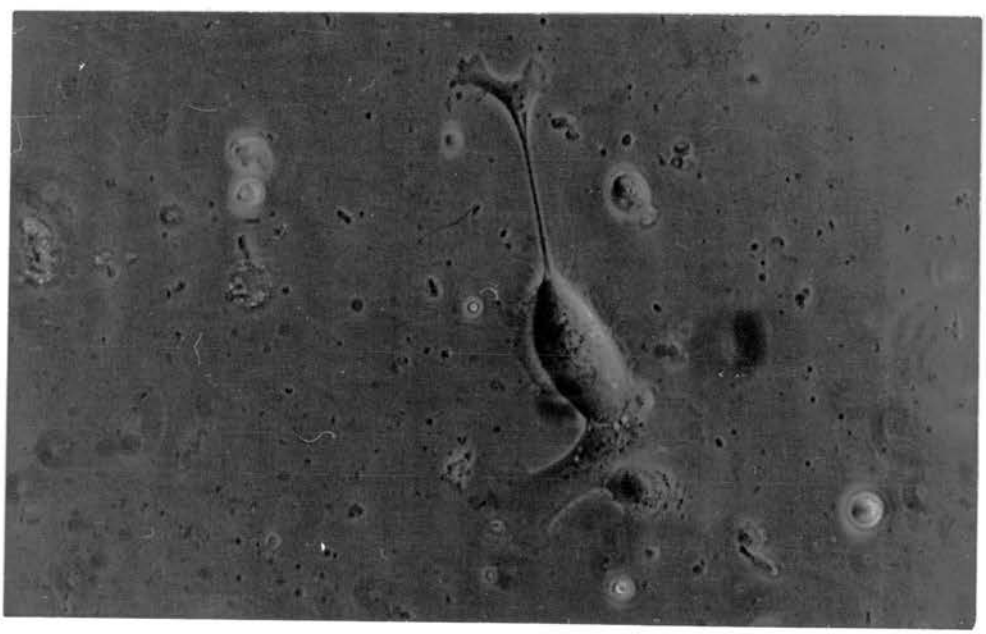
Oral squamous cell keratinocytes passaged through one subculture (x200 mag).

FIG. 43

Isolated group of keratinocytes derived from oral squamous cell carcinoma extending filipodia, lamellopodia and microspikes (x1000 mag).







keratinocytes by the extension of pseudopodia. Figure 43 shows an isolated group of tumour cells displaying filopodia, lamellopodia and microspikes.

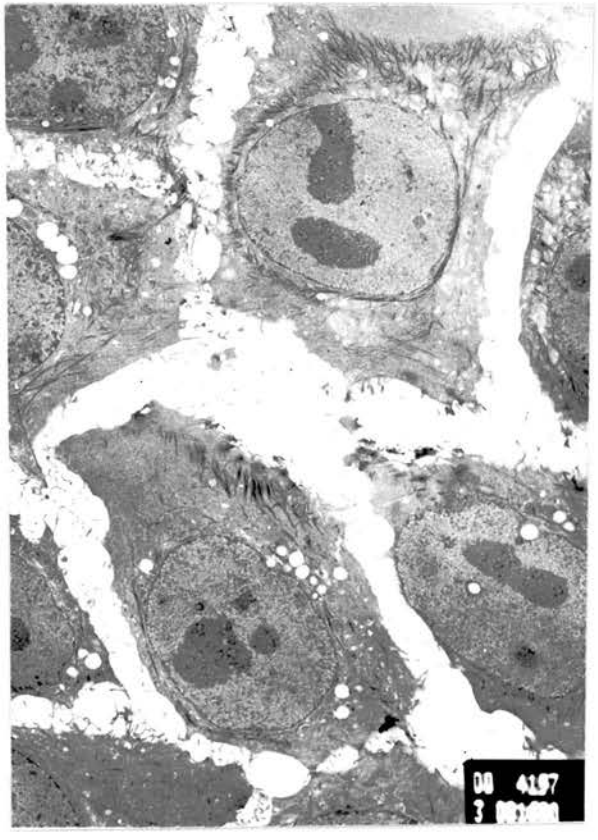
Under the electron microscope the epithelial origin of the tumour cells was confirmed. Figures 44-46 show tangential sections through three of the cultured tumours. The cells possess cytokeratin tonofilaments and display desmosomal junctions with adjacent cells.

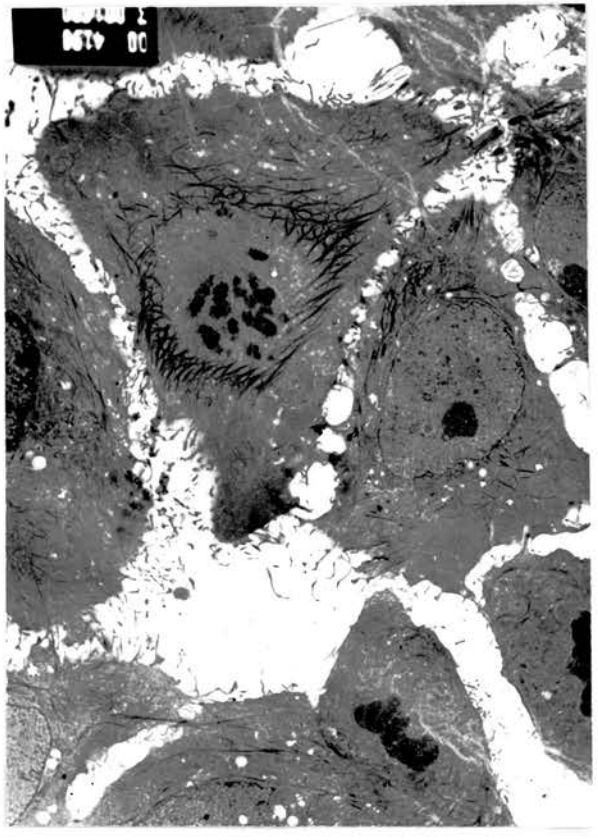
Nuclei of malignant cells were often markedly irregular with frequent invaginations of the nuclear membrane. This was not a consistent feature of all the cultured tumours examined but tumour cell nuclei very often had irregularities in shape (Fig. 46) and were sometimes very bizarre (Fig. 47), with pseudoinclusions not uncommon. Figure 48 shows a nucleus containing cytoplasmic contents, the inclusion being partly membrane bound although some cytoplasmic membrane is lost due to the angle at which the section was cut. This pseudoinclusion contained granules in the cytoplasm which were probably lipid filled, some of the lipid being lost during fixation. Ribosomes and endoplasmic reticulum were also present.

Two forms of chromatin with different densities were found in nuclei; a dense heterochromatin and an electron lucent euchromatin. Although the distribution varied from one cell to another, little difference was observed when cultured normal and malignant cells were compared (Figs. 33 and 44). Chromatin granules, which are found in a number of tumour cells, were not identified in cultured oral

FIGS. 44-46

Sections through keratinocytes from cultured oral squamous cell carcinomas from three patients (x2,100 mag).





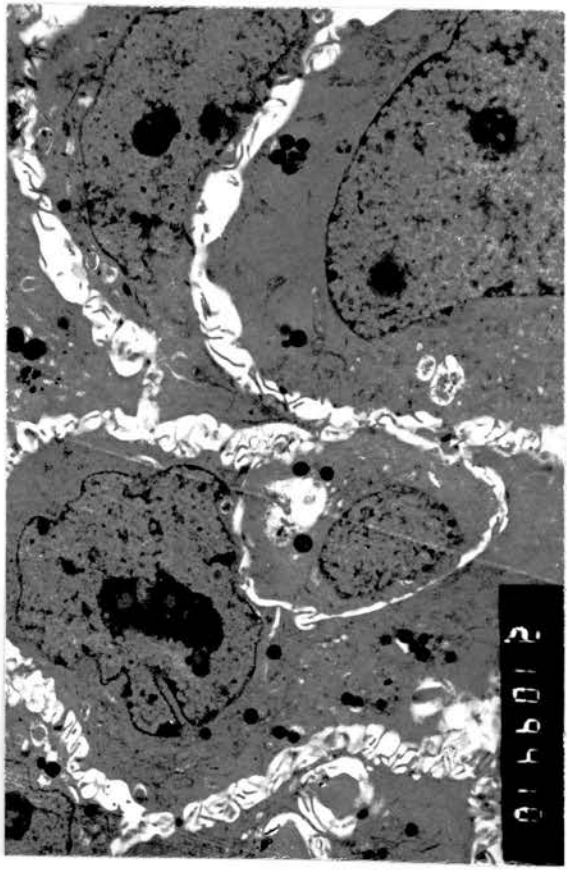


FIG. 47

Highly invaginated nucleus of cultured keratinocyte derived from oral squamous cell carcinoma (x5,600 mag).

FIG. 48

Pseudoinclusion in the nucleus of a cultured keratinocyte from oral squamous cell carcinoma (x7,000 mag).

Sb 10302

30 41K5
3 007100

carcinomas.

Increased numbers of nucleoli with marked irregularities in shape and size were seen in a number of cultures (Figs. 44 and 46). Nucleolar margination which facilitates cytoplasmic exchange also frequently occurred in tumours (Fig. 44). Many of the tumour cells frequently had irregularly shaped and swollen mitochondria with flooding of both matrix and intracristal space (Fig. 49). This feature was never observed in cultures of non-neoplastic epithelium. Desmosomes were present in cells in all cultured tumours, but when compared with normal cells, the desmosomes had a reduced complexity with little or no tonofilament structure (Fig. 50).

Generally the cytoplasm of cultured tumour cells contained ribosomes, rough endoplasmic reticulum and lysosomes. As in normal cultured epithelium, no Golgi bodies were found.

Tumour cell populations, like normal cultured keratinocytes were essentially pure epithelial cell outgrowths. Based on the presence of desmosomes and tonofilaments, almost 97% of the cells identified in randomly selected grids were keratinocytes (Table 13). Occasional leucocytes were seen migrating through the tissues.

2.7.3.3 Cytokeratin Staining

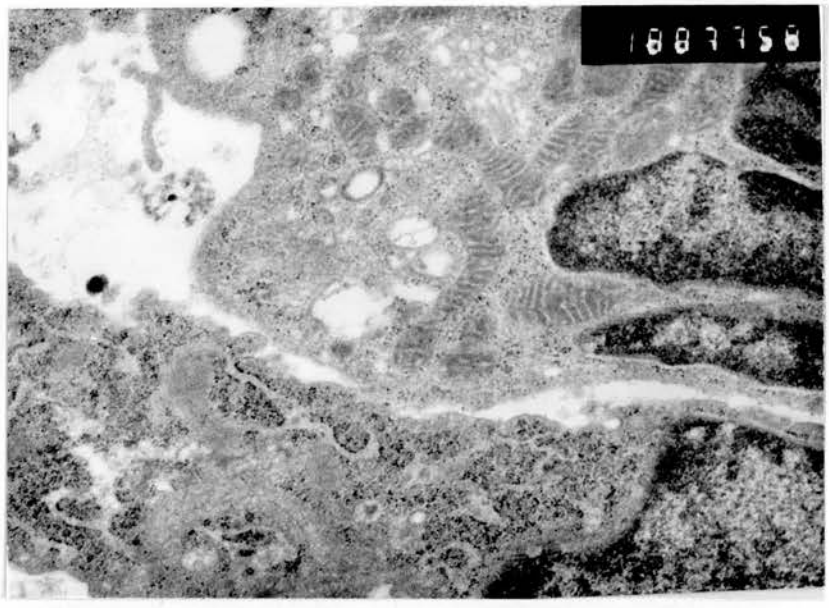
Not all tumour cell cultures could be examined by electron microscopy and because of the variation in cellular

FIG. 49

Mitochondria within cultured oral squamous cell carcinoma are typically pleomorphic and swollen, with flooding of the intracristal space (x18,000 mag).

FIG. 50

Poorly developed desmosomal attachments between cultured cells derived from oral squamous cell carcinomas (x35,000 mag).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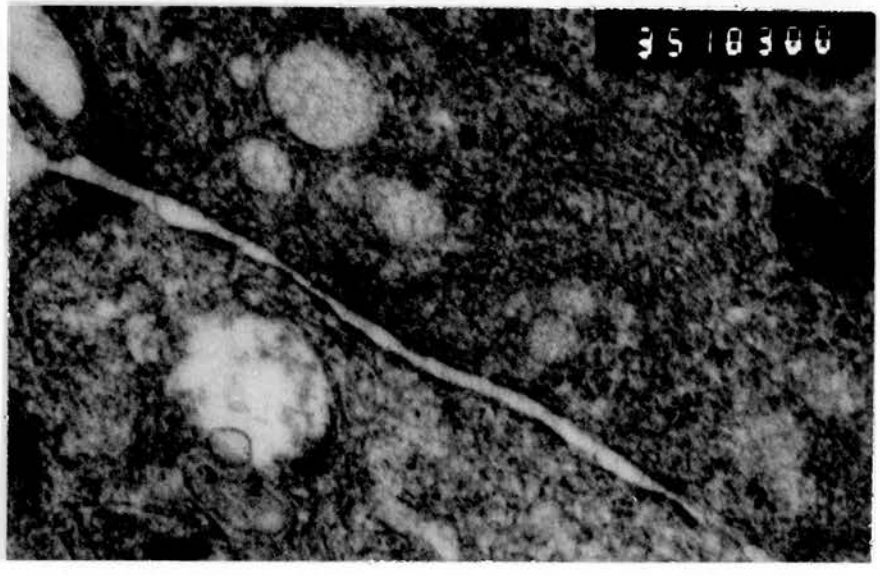

351800

TABLE 13

IDENTIFICATION OF CELL TYPES IN EM SECTIONS OF CULTURED ORAL SQUAMOUS CELL CARCINOMAS

Cell Types	Keratinocytes %	White Blood Cells %	Macrophages %	Mast Cells %	Fibroblasts %	Unidentified %
Biopsy						
1.	91.1	0	2.9	0	0	5.9
2.	99.4	0	0	0	0	0.6
3.	97.4	1.2	0	0	0	1.2
4.	99.3	0	0	0	0	0.7
Mean	96.8	0.3	0.7	0	0	2.1

n=460

morphology of cultured tumour cells at least one culture dish per patient was stained for cytokeratin using an immunoperoxidase staining techinique. All of the cultures examined in this way stained positively for cytokeratin. In each culture almost all of the cells stained with cytokeratin antibody (Fig. 51) although a small population of unidentified cells showed equivocal staining (Fig. 52). Cultures containing only fibroblasts were grown concurrently and all failed to stain with the cytokeratin antibody.

2.7.3.4 Autoradiography

In cultures the mean grain density over control cells incubated without thymidine was 1 grain per nucleus. Less than 0.1% of the cells had 5 or more grains over a nucleus and therefore a detection limit of 6 grains per nucleus was set which constituted a labelled cell (Fig. 53).

During the four week culture period, the thymidine labelling indices in cultures of normal gingival epithelium ranged between 8.9% and 12.9% with a mean labelling index of 11.4% (Table 14, Fig. 54). Between four and six weeks in vitro the thymidine incorporation into S phase cells fell to 0.5%. Little cell division was apparent in these cultures on visual inspection.

In tumour cutures TLI's ranged from 13.0% to 35.9% with a mean value of 21.6% +/- 3.3% (Table 15, Fig. 54). Tumour cultures had significantly higher TLI values (Mann-Whitney U Test p=0.006) than cultures of normal gingival epithelium. No attempt was made using this small sample size to

FIG. 51

Cultured keratinocytes from oral squamous cell carcinoma, stained with a polyclonal antibody raised to human cytokeratin (x100 mag).

FIG. 52

Population of cultured cells derived from oral squamous cell carcinoma showing different morphology with dendritic cells showing equivocal staining with polyclonal antibody raised to human cytokeratin (x100 mag).

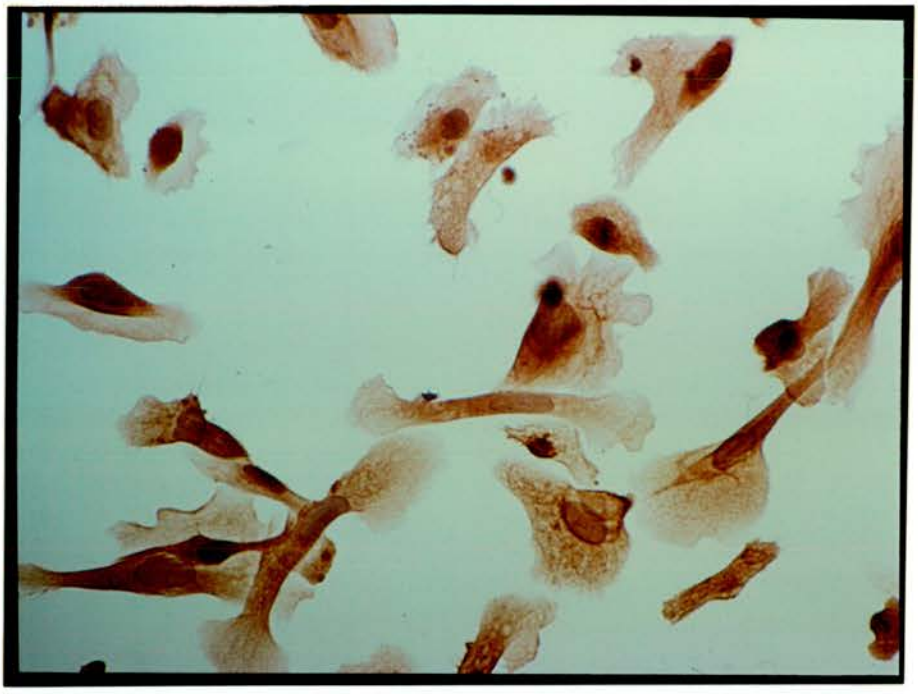
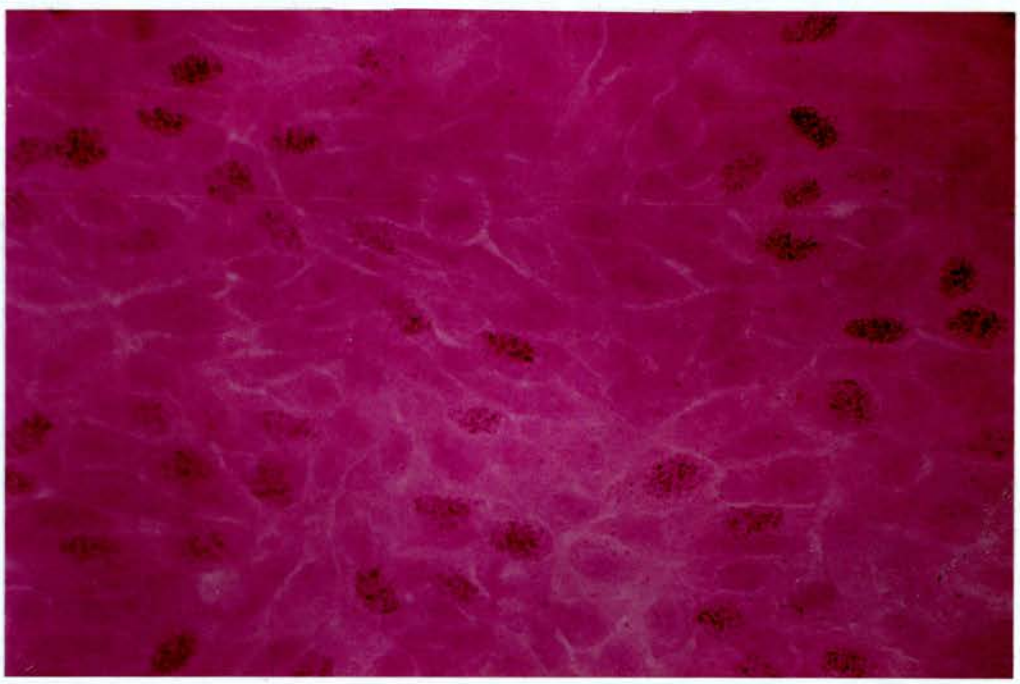


FIG. 53

Autoradiograph of cultured keratinocytes. Silver grains cover the nuclei of labelled cells (x200 mag).



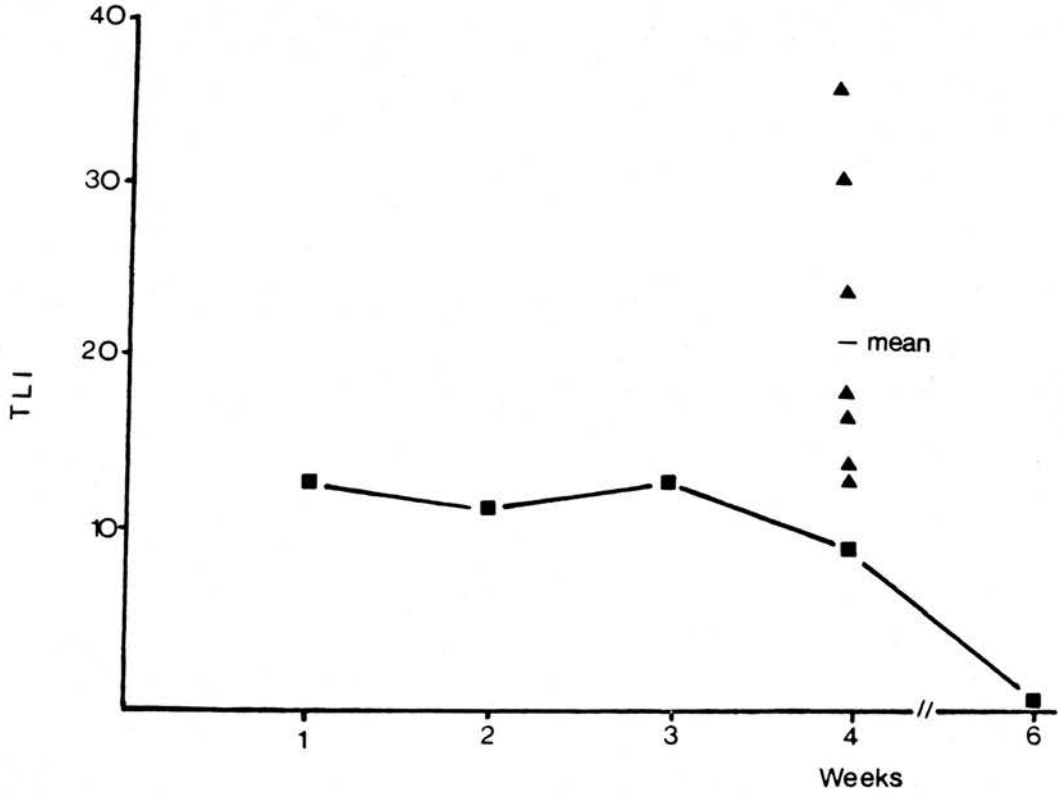


Fig. 54 **Thymidine labelling indices (TLI) of normal oral keratinocytes** (■ - ■) **and oral squamous cell carcinomas** (▲) <u>in vitro</u>.

TABLE 14

THYMIDINE LABELLING INDICES (TLI) OF CULTURED GINGIVAL KERATINOCYTES

Time In Vitro	1 wk	2 wk	3 wk	4 wk	6 wk
TLI (%) + S.E.	12.5 + 1.2	11.2 + 1.6	12.9 + 0.9	8.9 + 2.0	0.5
Number Of Cells Counted	2,981	21,407	7,436	8,867	1,750
Number Of Biopsies	4	4	4	4	2

TABLE 15

THYMIDINE LABELLING INDICES (TLI) OF CULTURED ORAL SQUAMOUS CELL CARCINOMAS AFTER 4 WEEKS IN VITRO

Biopsy	Labelled Cells	Total Cells	TLI (%)
T 3	734	2046	35.9
T 9	81	580	13.9
T 25	102	1352	13.0
T 28	150	903	16.6
T 32	1275	4160	30.6
T 36	1341	7548	17.8
T 37	1226	5146	23.8
Mean			21.6±3.3

correlate variations in TLI of tumours with patient age or sex or staging of the tumour (Table 16).

2.7.3.5. Karyotype

In cultures of normal gingival epithelium seventeen of the metaphase spreads had a normal chromosomal number (Figs. 55-57). Two of the twenty metaphase spreads were found to be hypomodal with a chromosome number of 45 (Fig. 58) and one metaphase spread had a hypermodal chromosome number (Fig. 59).

The several attempts made to obtain good metaphase spreads from oral tumour cultures all resulted in poor spreads with marked condensation of the chromosomes. Reduction in the time in which cells were incubated in colchicine to synchronise the cultures, and shorter incubation time in hypotonic solution to swell nuclei prior to spreading, failed to resolve these problems.

Conclusion:

Keratinocyte cultures from gingival epithelium retained features associated with the parent tissue. Keratinocytes migrated from explanted tissue and underwent cell division forming an ordered stratifying epithelium *in vitro*. Thymidine labelling indices of gingival epithelial cultures remained constant during the first four weeks of growth but decreased as cells senesced after six weeks *in vitro*. Polyploid cells were noted in cultures of normal gingival

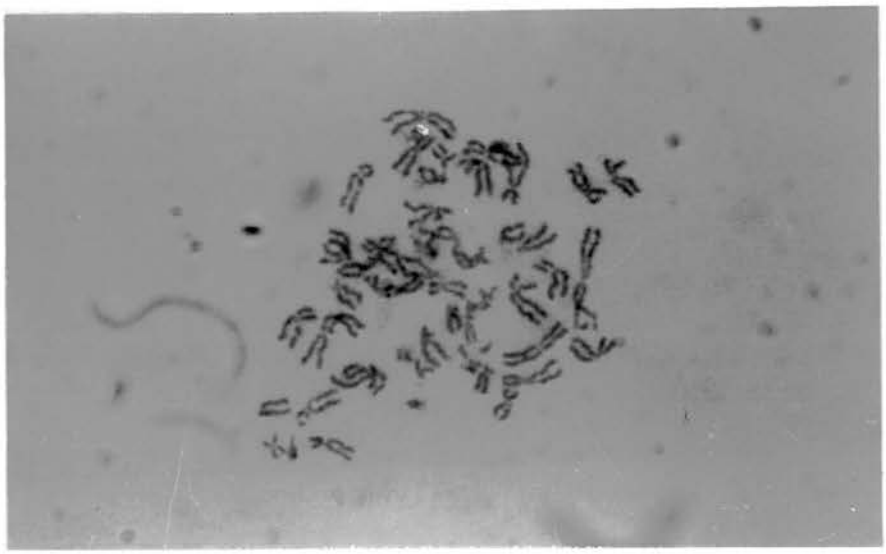
TABLE 16

HISTOLOGY OF TUMOUR SPECIMENS USED IN MEASUREMENT OF THYMIDINE LABELLING INDICES IN VITRO

Sample Number	Age	Sex	Site	Histology
T3	72	F	Cheek	Poorly differentiated squamous cell carcinoma of buccal mucosa
T9	78	F	Tongue	Poorly differentiated squamous cell carcinoma of tongue
T25	56	F	Tongue	Well differentiated, Keratinizing squamous cell carcinoma of tongue
T28	69	M	Buccal mucosa	Well differentiated, keratinizing squamous cell carcinoma
T32	58	F	Mouth	Well differentiated, keratinizing squamous cell carcinomas. Site of origin uncertain as carcinomas extensive
T36	72	F	Alveolar mucosa	Squamous cell carcinoma arising in floor of mouth and alveolar mucosa
T37	78	M	Tongue	Carcinoma-in-situ developing into squamous cell carcinoma of tongue

FIGS. 55 AND 56

Metaphase spreads from keratinocytes cultured from gingival biopsies (x1000 mag).



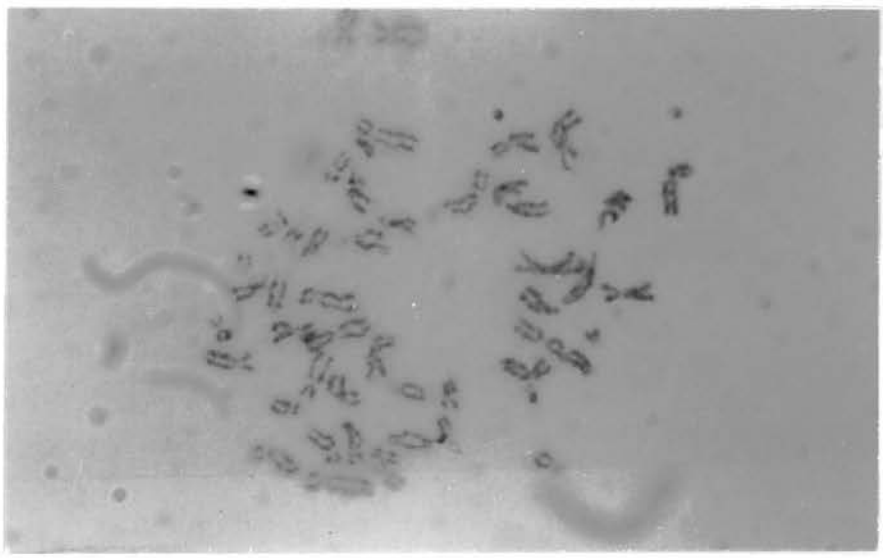


FIG. 57

Paired chromosomes from keratinocyte cultured from gingival biopsy showing normal chromosome constitution (x2,000 mag).

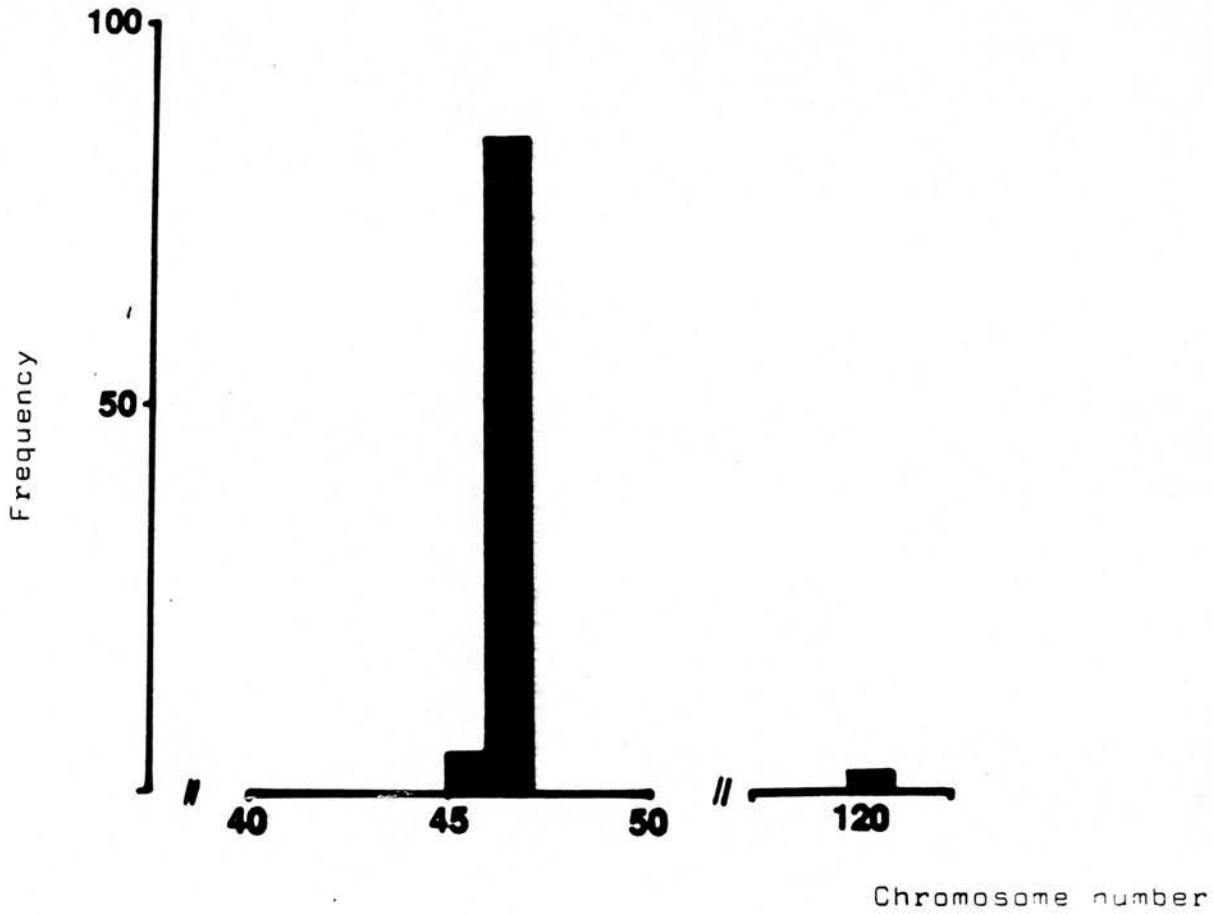
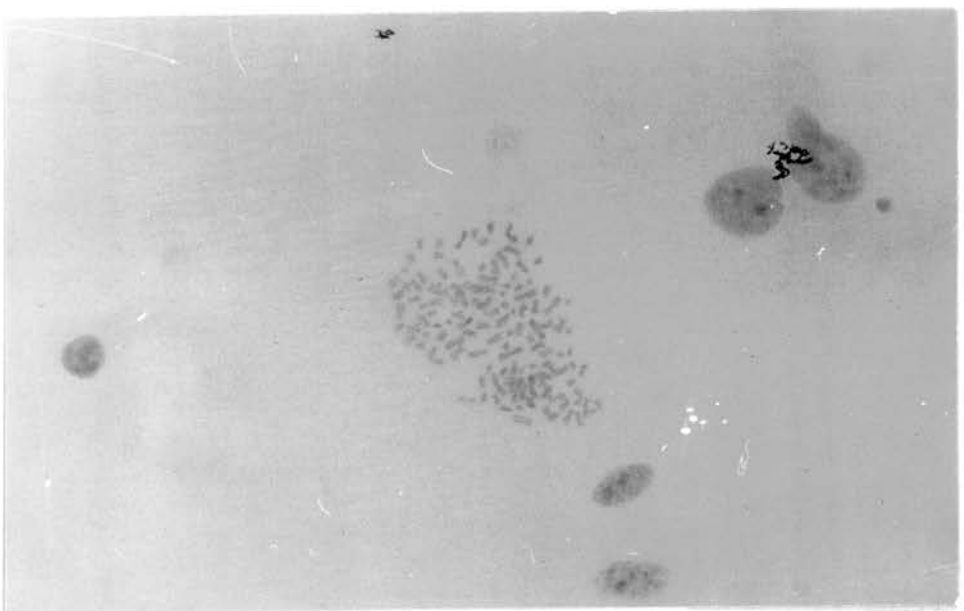


FIG. 58

Chromosome number in twenty representative oral keratinocytes cultured from gingival mucosa.

FIG. 59

Hypermodal chromosome number found in keratinocyte cultured from gingival biopsy (x200 mag).



epithelium but this may simply be related to a failure of the cells to divide.

A spectrum of subcellular changes was observed in epithelium cultured from oral squamous cell carcinomas. These changes were similar to those previously observed by others in histological studies of tumour sections. Thymidine labelling indices in tumour tissue varied but were generally higher than in cultures of normal gingival epithelium.

2.8 DISCUSSION

2.8.1 Keratinocyte Culture

The in vitro growth of normal human keratinocytes has tradionally been very difficult, due both to the inadequacy of available media and the problem of fibroblast overgrowth. Researchers initially overcame these difficulties by the use of lethally irradiated or Mitomycin C treated 3T3 feeder layers (Rheinwald and Green, 1975; Rheinwald, 1981) which probably served several functions. The growth rate of cells in culture is subject to two distinct influences. At low cell densities, growth rate commonly increases with cell density. Different mechanisms may contribute to this "feeder effect" (Gaunt and Subak-Sharpe 1977) including both contact with other cells or the extracellular matrix secreted by them, and by the diffusion of molecules from feeder cells. These molecules may be specific growth factors or simple compounds such as non-essential amino acids which may be lost from cells if there is an appreciable concentration gradient in low density cultures. The presence of lethally treated feeder cells also results in the inhibition of contaminating fibroblasts in epithelial cell suspensions, preventing the overgrowth of epithelial cultures by mesenchymal cells.

The growth of oral keratinocytes on feeder layers was investigated in the present study. Treatment of 3T3 feeder cells, which had grown to 50-75% confluence, with concentrations of Mitomycin as low as 3.75 $ugml^{-1}$ was found to inhibit proliferation of the cells without being toxic to

them. DNA synthesis continued although the cells were incapable of completing cell division. After 7-10 days these cells became granular in appearance with pyknotic nuclei and their detachament from the surface of the culture flask was apparent. After this time, however, replicating keratinocytes would normally be expected to have replaced 3T3 cells on the surface of the culture. Cell harvests from gingival biopsies were, due to the quantity of available tissue, low and, of the cells harvested by enzyme digestion, only a proportion were capable of further division. Bearing this limitation in mind, it was found that, even with seeding densities of $5 \times 10^4 - 1 \times 10^5$ cells cm^{-2} gingival keratinocytes were incapable of forming colonies which would maintain replication for a sufficient time for coalescence to occur.

A further problem relating to the use of feeder cells in the present study arose from the influence of dermal components on keratinocytes. It has recently been reported that co-cultivation of plasminogen activator producing cells, particularly with cells of mesenchymal origin, can result in the disappearance of plasminogen activator from the culture medium (Hoal et al 1983; Liu et al 1984). This may be the result of the production of protease nexins by mesenchymal cells (Farrell and Cunningham, 1986), which bind covalently to serine proteases leading to their internalisation and subsequent degradation. Certain other cell types such as human lung fibroblasts, virally transformed cell lines and

endothelial cells (Sprengers and Kluft, 1987) have also been found to produce fast acting inhibitors of plasminogen activators. Therefore, despite the fact that the feeder cells in successfully generated keratinocyte cultures are short lived *in vitro*, their effects on the production of plasminogen activator by keratinocytes are unclear.

Other modulating effects of 3T3 cells also remain largely unknown. In murine species connective tissue influences on epithelia have recently been established. Mackenzie and Dabelsteen (1986) found that in heterotypic recombinants of epithelia and connective tissue from various sites, an altered expression of blood group antigens on epithelia occurs in some matches. Sub-epithelial tissues, are, therefore, capable of signalling redirection of the pattern of phenotypic expression and some epithelia are capable of responding. Whether 3T3, or any other feeder cells, are capable of signalling such changes to keratinocytes in culture is uncertain.

Growth arrested feeder layers are not an absolute requirement for the growth of all human keratinocytes, particularly if the media and conditions of pH, temperature and seeding density are optimised. Several procedures for growing replicative cultures of different types of human keratinocyte have been reported (Stanley and Parkinson, 1979; Eisinger *et al* 1979; Lechner *et al* 1983; Assilineau *et al* 1986; Arneholt-Bindslev *et al* 1987). However, all of these require high seeding densities, usually in the order of magnitude of 10^5 cells cm^{-2} (Eisinger *et al* 1979) and reports

of keratinocyte growth at low seeding densities in the absence of feeder layers are still rare (Tsao *et al* 1983; Boyce and Ham, 1983).

Relatively few studies have been published on the *in vitro* growth of human oral keratinocytes (Mlinek and Buchner, 1975; Birkedal-Hansen *et al* 1980; Gusterson and Monaghan, 1979; Taichman *et al* 1979; Arneholt-Bindslev *et al* 1987) and most of these authors have relied on the generation of primary cultures from explants grown in a basal medium such as MEM, DMEM or M199 supplemented with antibiotics and 10% serum.

In the present study, explant culture was investigated as a method of cultivation of oral keratinocytes and it was found that the provision of MEM containing 10% serum in explant cultures of gingival mucosa resulted in significant fibroblast contamination after 2-3 weeks growth *in vitro*. Methods used to remove fibroblasts from these cultures were unsuccessful and a medium was developed which would favour keratinocyte growth and inhibit fibroblasts.

In developing a medium for this work two problems had to be resolved. Firstly, overgrowth of epithelium by fibroblasts had to be limited because of the potential production by fibroblasts of protease nexins or fast acting inhibitors of plasminogen activators. Secondly, many growth factors and hormones commonly added to cultures, in order to selectively stimulate keratinocyte growth, markedly affect plasminogen activator synthesis (Vassalli *et al* 1976; Rifkin,

1978; Lee and Weinstein, 1978; Gross et al 1983 a and b; Sudol, 1985) so requiring their use to be selective. Reduction of the serum concentration to 5% of the total volume of medium was sufficient to inhibit fibroblast growth, but growth of keratinocytes was characteristically slow producing small epithelial colonies approximately 1 cm in diameter. A number of growth factors and hormones were therefore considered for inclusion in the medium.

Steroid hormones, particularly hydrocortisone have been shown to markedly stimulate keratinocyte growth in vitro (Macaig et al 1980; Peehl and Ham, 1980 a and b; Tsao et al 1982). However, hydrocortisone and its synthetic analogues have been shown to inhibit biosynthesis of uPA and tPA by cells in vitro (Rifkin 1978). Induction, by hydrocortisone, of an intracellular regulatory molecule which inhibits either plasminogen activator gene transcription or mRNA translation is thought to be involved in this process. This effect may be prolonged requiring up to 72h for recovery of plasminogen activator synthesis by cultured cells (Rifkin, 1978). The use of hydrocortisone was therefore excluded. It is generally agreed that cAMP is the secondary messenger for these hormones. Cholera toxin which increases cAMP levels in cultured cells has been shown to stimulate tPA production by Schwann cells in vitro (Alvarez-Bullya and Valinsky, 1985). The long term effect of cholera toxin on PA production has not been documented and its use was not investigated in the present study.

Of the polypeptide hormones, EGF is a common addition

to keratinocyte cultures (Eisinger et al 1979; Macaig et al 1980; Rheinwald, 1981; Tsao et al 1982; Boyce and Ham, 1983) and optimum stimulation of proliferation of these cells has been reported with nanogram quantities of EGF. Down regulation of EGF receptors following EGF treatment of cultured cells is correlated with an increase in plasminogen activator activity (Lee and Weinstein, 1978; Gross et al 1983a; Hamilton et al 1984). Using the epidermoid carcinoma cell line A431, Gross et al (1983b) reported that removal of EGF from previously down regulated cells resulted in recovery of total cellular EGF binding activity and concurrent loss of plasminogen activator activity. Plasminogen activator levels in these cultures began to fall immediately on the removal of EGF with half maximal loss within 5h. Such a rapid response to the removal of EGF from these cultures did not preclude its use in this study.

The addition of EGF ($10ngml^{-1}$) to MEM containing 5% serum was sufficient to achieve a more rapid and prolonged growth of gingival keratinocytes in explant culture. Keratinocytes grew more readily in this medium than in medium containing 10% serum alone. Both these media were, however, sub-optimal since, in medium containing 10% serum which was further supplemented with EGF, greater growth of keratinocytes was achieved than in medium with 5% serum and EGF or 10% serum alone. Two other additives, with no reported effects on plasminogen activator synthesis, were tested for use in conjunction with EGF. Insulin is

stimulatory for a large number of cells *in vitro* (Mather *et al* 1980) but its use in gingival keratinocyte cultures produced only a small increase in colony growth. Insulin is normally used in culture at levels far in excess of physiological concentrations because it is rapidly inactivated at 37°C, particularly in serum free medium. This may account for its limited effect in these cultures. One further supplement, phosphoethanolamine, was tested in conjunction with EGF and insulin at a concentration previously reported to stimulate foreskin keratinocyte growth in MCDB152 medium (Tsao *et al* 1982). In the present study, this mixture of additives was found to be inhibitory to keratinocyte growth when compared to medium containing only EGF and 5% serum. Fibroblast contamination had also increased in this more complex medium with over 29% of the colonies showing fibroblast contamination compared with less than 10% in medium containing only EGF. Using a point counting method, fibroblast contamination was estimated in the first twenty keratinocyte cultures grown in EGF containing medium. The areas of culture dishes covered by fibroblasts remained low with a median value of 0.5%. Therefore, this medium was utilised in all subsequent experimentation.

As expected reduction of serum from 10% to 5% reduced the plating efficiency of explanted gingival tissue *in vitro*. In practice, however, the reduction was too small to consider the use of coated substrates. Between 60-70% of the explants regularly attached to the substrate and formed epithelial

colonies, this being sufficient to give good coverage of the culture dish.

Using this method just over 50% of oral squamous cell carcinoma biopsies were successfully grown in explant culture. This compares favourably with results in other laboratories (Johns et al 1982; Schiff and Schugar, 1984; Mattox et al 1984). Mattox et al (1984) found that only 36% of head and neck tumours grew in a soft agar cloning assay - 35% failed to grow and 29% were contaminated. Johns et al (1982) achieved a slightly higher success rate with 51% of their head and neck tumours producing epithelium, but again there was a high contamination rate. As these authors found, contamination of biopsy material was a major factor in the failure to grow explant cultures of oral carcinomas in the present study. Even in the absence of contamination, however, fewer tumour specimens than gingival specimens were cultivated in vitro. Often many of the cells within a tumour mass are not viable, perhaps as a result of anoxia or previous therapy, and further cell losses probably occur during surgical procedures. Investigation of improved media and culture conditions for growth of these tumours in vitro is essential. Optimising media for tumours is complicated by autocrine secretion of growth factors by tumour cells (DeLarco and Todaro, 1978). This may vary from tumour to tumour and the addition of exogenous growth factors, including those present in serum, may actually inhibit growth in vitro.

In the present study, passage of oral keratinocyte cultures was singularly unsuccessful. Subculture of keratinocytes from primary cultures in split ratios as low as 1:1.5 failed to produce confluent cultures. Other workers growing oral keratinocytes have reported similar findings. Taichman et al (1979) reported passage of normal oral keratinocytes through two to four subcultures using the 3T3 feeder technique, with an estimated cloning efficiency of 1.5%. Without feeders, cloning efficiency dropped to 0.002%. Recently Arneholt-Bindslev et al (1987) developed a new subculture technique. They found that passaging cells in a 1:2 split ratio was unsuccessful, but if secondary cultures were initiated using a drop culture technique, with seeding densities of 10^5 cells per 20 ul drop, successful subculture through six passages could be achieved. This finding is important since it allows experimentation with passaged cultures without the inconvenience of inter-patient variation.

Passage of keratinocytes derived from oral squamous cell carcinomas was less difficult. In three cases serial cultivation of tumours was achieved through 2 - 4 passages. Again the production of endogenous growth factors by tumour cells may provide essential elements lacking in the tissue culture medium. Improvement of cell culture conditions and media for these cells must be a priority since both the long and short term requirements of the cells have not been satisfied.

Boyce and Ham (1983) reported that human foreskin

keratinocytes could be serially cultured through 7 passages in MCDB 153 with seeding densities as low as 400 cells cm^{-2} in the absence of 3T3 cells, conditioned medium, surface coatings or serum. Fibroblasts are reported to be inhibited in this medium. Such low seeding densities could be of considerable value in the culture of oral keratinocytes where biopsy material is often limited. There is nothing intrinsically novel about this medium which contains water, salts, buffers, bulk organic ions, trace elements, amino acids and a number of organic substances to replace serum. However, even this complex medium cannot be considered minimal. It is not known, as yet, whether the components of this medium fully satisfy all the short term growth requirements of all keratinocytes. Although serial culture can be achieved at low seeding densities in MCDB 153, cells can still only be passaged through a very limited number of subcultures before senescing. Long-term requirements of keratinocytes in this medium have still not been met.

Of interest is the defined nature of the medium which allows precise investigation of drugs or hormones *in vitro* in the absence of undefined supplements which may interfere with the action of the agent studied. Primary or passaged cells grown in this medium do not, however, produce cultures which are a good *in vitro* model for skin. Low calcium concentrations in this medium shift the normal pattern of differentiation, maintaining unstratified cultures. The addition of calcium and serum does induce differentiation

while in their absence proliferation of cells is optimised. Indeed, despite the quality of the medium, since its original description in 1983 it has been adopted by very few other laboratories (Cuono et al 1986; Pittlekow and Scott, 1986) for keratinocyte culture. Problems of water purity, the cost of tissue culture grade components, and the complexity of the method for making the medium have obviously not been resolved in other laboratories.

2.8.2 Characterisation of Oral Keratinocytes In Vitro

Epithelium in vitro grows with a distinctive morphology. Cells plated either as single cell suspensions, or outgrowths from explanted tissue produce cultures with a typical pavement-like structure and the epithelium eventually forms a confluent polarized ordered epithelium.

The structure of primary cultures of human oral keratinocytes was typical of cultured keratinocytes grown in other systems (Gusterson and Monaghan, 1979; Marcelo et al 1980; Arneholt-Bindslev et al 1987), and over 90% of the cells in vitro contained conspicuous cytokeratin tonofilaments and desmosomes - structures only associated with epithelial cells. Basal cells which attached to the culture dishes retained a more squamous appearance than basal cells in vivo. Supra-basal cells showed varying degrees of differentiation which was characterised by an increased accumulation of cytokeratin tonofilaments, a reduction in the number of desmosomes and lysis of subcellular organelles. Occasionally accumulations of keratohyalin granules were

observed in cells growing just beneath the sloughing layer, indicating a similar pattern of organisation to that expressed *in vivo*.

Tumour epithelial cells, cultured in the same conditions as normal oral keratinocytes, varied markedly in their degree of differentiation, probably reflecting the heterogeneity of tumour populations and their growth pattern prior to explantation. Again over 90% of the cells cultured from tumour explants featured either desmosomes or cytokeratin tonofilaments or more usually both, thus confirming the squamous origin of the tissue. All tumour cultures, however, showed irregularities not associated with the growth pattern of normal cells *in vitro*. Increase in the size and irregularity of nuclear shape is a common feature in histological sections of tumour (Ghadially, 1975). In the present study, many cultured cells showed highly invaginated nuclei, which often resulted in the appearance of pseudo-inclusions in the nuclear matrix. Similarly nucleolar alterations occur in tumour cells, and an increase both in the size and numbers of nucleoli within the nuclei of cultured oral squamous cell carcinomas was observed. Nucleolar margination, often associated with intense synthetic activity, was frequently seen in cultured tumour cells. The deep invagination of the nuclear membrane may be another form of nucleolar margination which facilitates nucleo-cytoplasmic exchange. The predominance of glycolytic metabolism *in vitro* generally reduces the numbers of

mitochondria observed in these cells. The oral squamous cell carcinomas cultured in this study were no exception. However, perhaps the most consistently observed abnormality in these cells was both the pleomorphism associated with mitochondria and flooding of the cristae in the mitochondrial matrix which has been observed in histological sections of tumours by others (Sordahl *et al* 1969; Pederson, 1970).

Tumour cells *in vitro* largely contained very rudimentary desmosomal complexes which reflected the poor adhesion observed between these cells in the light microscope. Few cells contained markers of differentiation with keratohyalin granules, Golgi complexes and rough endoplasmic reticulum being sparse. These ultrastructural abnormalities indicate a departure from normality. Similar changes were also observed in epithelium cultured from the excision margins of tumours in which epithelium appeared macroscopically normal. These subcellular changes may prove to be a useful indicator of the "normality" of cells at the excision margins of tumours.

2.8.3 Thymidine Labelling Indices

The thymidine labelling indices, as a measure of the proliferative activity of normal cultured oral keratinocytes remained relatively constant during the first four weeks *in vitro* with TLIs ranging from 8.9-12.9%. After six weeks there was a marked decline in the thymidine labelling index which was probably related to senescence of the cells.

Albers et al (1986) similarly found using both autoradiography and flow cytometry that 8% of their keratinocytes were in the proliferative pool. Gusterson and Monaghan (1979) also reported a sharp decline in the mitotic activity of their oral buccal keratinocytes in explant culture after 6 weeks in vitro, but their initial TLI value was higher (27%) after a 2h pulse label. Marcelo et al (1980) reported TLI values of between 10-30% for cultured mouse keratinocytes over a period of 2-25 days in vitro. Their labelling period of 12h was considerably longer than that used in the present study (1h), and probably encompassed populations of cells entering into more than one S phase of the cell cycle in the labelling period.

Incorporation of 3HTdR showed a focal distribution in the oral keratinocytes. This confirms the observations of Gusterson and Monaghan (1979), but is contrary to the report of Karasek et al (1966). The most widely accepted model of epidermal cell renewal is that of Potten (1981) who envisaged two classes of replicating cells. He argues that stem cells are capable of extensive renewal and amplifying cells, which arise from stem cells, undergo limited division before terminal differentiation. Cell division in oral epithelium in vivo is also not evenly distributed thoughout the basal layer but occurs in regular clusters (Squier et al 1976). In epithelium with a complex connective tissue interface such as attached gingiva, cell division often occurs in clusters at the tips of rete ridges. Focal areas of mitotic activity of oral keratinocytes in vitro and in vivo would accomodate the

EPU model of Potten.

Unlike normal cultured oral epithelium, tumour cell labelling was not focally distributed. Focal areas may have been more difficult to visualise in very actively replicating cultures, but even in tumour cultures with lower TLIs, focal labelling was still not readily apparent. Cultured tumours had significantly higher TLI values than normal cultures but too few oral squamous cell carcinomas were grown in the present study to correlate histological staging, patient age or sex with tumour growth. However, it is interesting that those patients with most frequent recurrences of tumours showed highest labelling indices (35.9 and 30.6) of cultured tumour cells *in vitro*. The release of tumour cells from normal growth constraints *in vivo* is therefore reflected *in vitro*. Tumour cells in the absence of modifying influences *in vivo*, continue to show an abnormally high turnover rate and this would suggest some permanent change in the underlying metabolism of the cells.

2.8.4 Karyotyping

Karyotypic analysis is of great importance in monitoring changes in chromosome constitution which occur in culture. In the present study, the karyotype of cultured gingival epithelial cells was normal in 17 of the 20 selected metaphase spreads. Two of the twenty cells were hypomodal, but it is uncertain whether this was due to inadequate preparation or whether this was a real phenomenon. Wenger *et*

al (1984) suggest that to rule out a rare event a Poisson probability can be used. Based upon a 95% confidence limit for a Poisson distribution of one event, the probability of finding two similar events require 0.015x = 2, where x = 19 cells (Rao et al 1966). Two non modal cells within this population is therefore highly significant, however, it was considered that larger numbers of cells should be screened to rule out hypomodality as a feature of cultured oral keratinocytes.

Hypermodality was identified in one cell in the metaphase spreads and was unlikely to result from inadequate preparation, particularly as the spread was well defined. Polyploidy has also been observed in cultures of human foreskin keratinocytes (Peter et al 1988). In their study tetraploid cells appeared after only two days in culture, and these increased in number from 10-25% of the cells present during the first month of culture (unpassaged). Although changes in chromosome number were found to accumulate during the early culture period, adapted populations of keratinocytes stabilised again at the diploid level after one to three months in culture. Structural chromosomal aberrations, mostly gaps and breaks, were common in cells throughout the whole culture period of three months.

In one study in which karyotpic analysis of keratinocytes grown from buccal mucosa in explant culture was performed, no chromosomal abnormalities were found in keratinocytes from five patients after 20 - 25 days in vitro

(Arneholt-Bindslev et al 1987). These authors, however, report the growth of oral keratinocytes at 34° C. Since Peter et al (1988) found that elevated culture temperature (40°C) enhanced the appearance of chromosomal abnormalities in foreskin keratinocyte cultures, it may be that growth of keratinocytes at lower temperatures reduces the frequency of chromosomal alterations.

The results in the present study agree with those of Peter et al (1988) but the functional significance, if any, of polyploid cells in these cultures in unknown.

The diploid chromosome constitution is the result of natural selection acting on the organism as a whole where complex demands must be met. In contrast, in tissue culture selective advantage operates simply in favour of the fastest growing cells. This may result in chromosome aberrations where omission of mitosis entirely (polyteny) results in DNA duplication cycles which follow one another or alternatively, aberrations in the mitotic process might result in the fusion of daughter nuclei. Such mitoses are termed polyploidizing. Brodsky and Uryaeva (1969) postulated that in culture metabolic resources are finite and therefore performance of some functions must be inhibited at the epigenome level. In the cultured tissues they investigated, they found that the molecular basis of polploidy resulted from competition of different metabolic pathways for a lacking metabolite or in translational competition. Whether this is the basis for polyploidy in cultured keratinocytes remains to be determined.

2.8.5 Summary

In the present study a medium was developed in which keratinocytes from gingival mucosa and oral squamous cell carcinomas could be grown in explant culture and which was suitable for use in studies of plasminogen activators in cultured cells. Reduction of the serum content of the medium and addition of EGF effectively controlled fibroblast growth in the majority of the cultures, which grew as relatively pure populations of epithelial cells. Some differentiation of gingival epithelial cells was apparent *in vitro*, and although less complex that *in vivo*, cells moving from the basal layer to the more superficial layers became elongated and accumulated keratohyalin granules and cytokeratin tonofilaments. A loss of subcellular organelles was also noted in superficial cells although complete dissolution of nuclei was not apparent.

Proliferation *in vitro*, assessed by thymidine labelling, was similar to that reported for other non-neoplastic keratinocytes and remained between 9-13% during the first month of culture. In karyotypic analysis of cultures, 5% polyploid keratinocytes were identified *in vitro*. This has been shown by others to be common in keratinocytes cultured from normal skin and that these cultures stabilise again at the diploid level.

Cultures were more difficult to establish from oral squamous cell carcinomas, than from normal gingiva. Once

established, however, the tumour cultures grew more readily *in vitro* and the thymidine labelling indices of tumours were much higher than those of gingival keratinocytes. Tumour cells could also be subcultured through at least 2-4 passages. In order to confirm that tumour cells retained features associated with malignant cells *in vivo*, cultured tumour keratinocytes were examined ultrastructurally. Tumour cells showed a spectrum of ultrastructural abnormalities, which have been observed in histological sections of tumours. These include invagination of nuclear membranes, increased frequency of pseudoinclusions, increased size and numbers of nucleoli, and reduced numbers and complexity of desmosomal junctions. In normal and neoplastic cultured cells mitochondria were generally reduced in number, but in tumour cells mitochondria were often pleomorphic and swollen, a feature never associated with cultured gingival keratinocytes. This tissue culture model was therefore suitable for the study of plasminogen activator synthesis by normal and malignant cells *in vitro*.

C H A P T E R 3

FIBRINOLYTIC ACTIVITY OF ORAL EPITHELIUM

3.1 INTRODUCTION

Plasminogen activator has been demonstrated by fibrinolytic autography and immunocytochemistry in epithelium in a number of sites from a variety of mammalian species. Plasminogen activator of an undetermined type has been shown by autography to be present in rat, guinea pig and rabbit epidermis (Smokovitis and Astrup, 1978), squamous epithelium of the uterine cervix (Todd, 1964), and in rat and human oral epithelium (Wunschmann and Astrup, 1974; Southam and Moody, 1981; Southam, Moody and Kowolik, 1981; Birkedal-Hansen and Taylor, 1983). More specifically, urokinase type plasminogen activator has been detected in epithelium from kidney tubule, ductus deferens, mammary gland and lung (Larsson et al 1984), from human prostate (Camiolo et al 1984) and from glomerulus and kidney convoluted tubule (Nakamura et al 1984; Angles-Cano et al 1985).

Functional studies on these enzymes, which are products of different genes, have indicated that tPA is primarily involved in the maintenance of fluidity in the extracellular environment, most notably in thrombolysis. uPA is considered to be responsible for localised proteolysis during cell migration and tissue remodelling in both normal and pathological processes (Dano et al 1985).

It has been suggested that keratinocyte plasminogen activator may facilitate the normal differentiation events of nuclear dissolution (Myhre-Jensen and Astrup, 1971; Green, 1977) and evidence that nuclear destruction is a plasmin assisted process supports this concept (Green, 1977; Ossowski et al 1979). Further evidence that plasminogen activators are involved in the terminal differentiation events of keratinocytes comes from in vitro studies. Isseroff et al (1983) and Morioka et al (1985) found that plasminogen activator activity produced by cultured keratinocytes increases at confluence, the greatest activity being found in cells consisting mainly of cornified envelopes shed from the culture. Other investigations have shown that uPA may also be involved in cell migration. Fibrin and fibronectin which are present under migrating cells of re-epithelialising wounds gradually disappear from the basement membrane after wound healing (Clark et al 1982) and it is possible that their degradation is the result of plasminogen activator activity in epithelial cells. Krystosek and Seeds (1985) have shown that cultured cells including neurons, a malignant neural line and transformed fibroblasts deposit plasminogen activator on growth substrata, including collagen coated plastics, where it is strategically well placed to participate in local degradation of components of the extracellular matrix. The finding by Morioka et al (1985) that uPA accumulates in wounded keratinocyte cultures at the newly formed wound edge within 16h supports the postulate that uPA is involved in cell migration and wound healing.

Both Morioka et al (1985) and Hashimoto et al (1985) demonstrated that the plasminogen activator produced in cultures of neonatal foreskins is predominantly of the urokinase type.

3.1.1 Plasminogen Activators in the Oral Cavity

Plasminogen activator activity of human oral epithelial cells was first demonstrated in cells obtained from buccal smears (Birn and Fejerskov, 1971; Wunschmann-Henderson and Astrup, 1972). Fibrinolytic autograph studies by Southam (1981) and Southam, Moody and Kowolik (1981) showed that while fibrinolytic activity could be demonstrated in intact oral epithelium it could not be demonstrated in relation to epithelium from all parts of the mouth; epithelium from dorsal and ventral tongue, the cheek, palate and gingival sulcus showed activity but gingival epithelium (and epidermis) did not show activity. The authors showed that lysis occurred on fibrin plates in potassium thiocyanate (KSCN) extracts of epithelia from all sites, although lysis did not occur in all the saline extracted fractions. These findings were in agreement with those of Albrechtsen (1958) and suggested that the activator enzymes in saline extracts of tissue represent those enzymes immediately available from a tissue in vivo and that saline extracts contain only the activator demonstrable by autography. The failure to demonstrate lysis in all sites with fibrinolytic autography was therefore simply a reflection of insufficient activator

present in the tissue for detection with the technique and not an inability of the tissue to produce activators. No functional differences in the levels of plasminogen activators in the various sites using human tissues were determined.

Birkedal-Hansen and Taylor (1983) have also demonstrated fibrinolytic activity in rat tongue epithelium by autography. From cultured rat tongue keratinocytes they separated three electrophoretically distinct activators - uPA (Mr≃48KDa = Mr≃55KDa in humans), tPA (70-75KDa) and a high molecular weight activator (95-105 KDa) which has been found in other epithelial cultures (Wilson et al 1980).

Human saliva also contains plasminogen activator which is derived from the cellular component. Soluble activator cannot be demonstrated in the supernatants of mixed, parotid or submandibular salivas (Moody, 1982) - resuspended salivary pellets showing fibrinolytic activity only in association with epithelial cells and cell fragments. Goldstein et al (1971) reported that normal viable polymorphs from peripheral blood contain a small amount of plasminogen activator, but leucocytes at concentrations present in saliva in a healthy dentulous mouth cannot produce measurable lysis on a fibrin plate. Fibrinolytic activity has been ascribed to bacteria present in the mouth, however, Moody (1976) showed that bacteria can at the most make only a minor contribution to the fibrinolytic activity of saliva.

3.1.2 The Role of Plasminogen Activators in Epithelium

The role of plasminogen activators in epithelium in relation to normal and disease processes has not been firmly established, but the following roles have been suggested.

3.1.2.1 Post-Operative Healing

The surgical observation that blood clots rarely persist in the oral mucosa is consistent with the idea that local fibrinolytic activity in the mouth is a feature of healing. Although no direct evidence is available, it is reasonable to assume the fibrinolytic activity in regenerating epithelium enhances the ability of the epithelium to grow beneath fibrin clots (Linjen and Collen, 1972).

3.1.2.2 Post-Extraction Infection

Material removed from post-extraction sockets shows significantly higher fibrinolytic activity in "dry sockets" than it does in normal healing sockets (Birn, 1970). This is thought to be due to activators released from alveolar bone rather than from the oral epithelium (Bjorlin *et al* 1986).

3.1.2.3 Chronic Periodontal Disease

Periodontal disease, the principal cause of tooth loss in adults, is characterised by gingival infiltration with inflammatory cells which ultimately results in the loss of connective tissue. Micro-organisms are the primary aetiological agents in the disease (Ellison *et al* 1970) and individual organisms eg. *Actinomyces viscosus*, when used

singularly as contaminants can produce the disease in germ-free rats (Guggenheim et al 1964).

The mechanisms by which plaque organisms can elicit inflammatory periodontal disease are not fully elucidated. Both antibody and cell mediated immune responses to the bacteria associated with plaque have been reported in man (Evans et al 1966), but whether or not these actually contribute to the pathogenesis of the disease is unknown.

Several bacteria, including Actinomyces viscosus and other plaque organisms, are among the agents which can activate complement by the alternative pathway. Activation of complement generates mediators of acute inflammation and factors chemotactic for polymorphs and macrophages are also generated (Alper, 1974). The component C3a is highly cytotoxic for a number of cell types, and could contribute to the fibroblast cytotoxicity which is an early feature of the periodontal lesion. Another cleavage product, C3b, induces the secretion of hydrolytic enzymes such as elastase, collagenase and other broad spectrum proteases from polymorphs and macrophages which could further contribute to tissue breakdown. Activation of the terminal components of complement leading to the synthesis of prostaglandin E also provides a mechanism by which bone resorption may occur.

In view of these findings a role for plasminogen activators in local pathological processes in gingival epithelium is not hard to envisage. Plasminogen activator was always found, by Southam et al. (1981), to be present in

gingival pocket epithelium on fibrinolytic autographs. Plasminogen itself is present in saliva (Moody, 1982) and a mechanism therefore exists for the generation of plasmin in the gingival crevice. Plasminogen is also present in extravascular gingival tissue and can permeate the oral mucous membrane (Brandtzaeg, 1973; Genco et al 1974). Thus plasmin, which is capable of activating complement by either the alternative or classical pathways, generated in this way provides another mechanism of tissue destruction. Plasmin generated in the gingival crevice can also activate latent collagenases which may intensify the disease process (Paranjpe et al 1980). In addition, plasmin released in the gingival crevice or pocket may also enhance the bleeding tendency by the production of fibrin degradation products which themselves have anticoagulant properties (Marder, 1971). Indeed a dialysable peptide fraction occurring as the result of fibrin degradation has immunosuppressive activity and this may further alter local tissue responses in gingivitis.

3.1.2.4 Pemphigus

Evidence suggests that the intra-epithelial bullae in pemphigus result from the pemphigus antibody stimulating keratinocytes to release plasminogen activator which then converts serum derived plasminogen to plasmin. The plasmin in turn digests the intercellular attachments between keratinocytes (Friedman, 1986). Morioka et al (1984, 1987) further showed that acantholysis induced in skin explants in

the presence of pemphigus IgG can be blocked by anti-urokinase antibody.

3.1.2.5 Malignancy

Tumour invasion and metastasis is a multi-stage process involving detachment of cells from the primary tumour, penetration of the basement membrane, and movement of cells via the blood or lymph system to the target organ. Epithelial tumours, in particular squamous cell carcinomas, must therefore be able to degrade basement membrane constituents and connective tissue. For many years tumour tissues have been shown to have substantial fibrinolytic activity which may be involved in extracellular proteolysis (Fischer, 1925; Goldhaber et al 1947; Astedt et al 1971). However, it is now well established that normal tissues from a number of anatomical sites - epidermis, bone, brain, uterus, ovaries, and testes (Dano et al 1985) produce plasminogen activators which are involved in normal physiological processes. What remains unclear is whether or not altered plasminogen activator synthesis, either qualitatively or quantitatively, is involved in the malignant potential of tumours. Some research has indicated elevated levels of plasminogen activators of a non determined type in extracts of tumour homogenates compared to the equivalent normal tissue (Nagy et al 1977; Chylak and Brdar, 1983; Soszka and Olszewski, 1986). However, tPA is found in endothelial cells of many blood vessels and is likely to be found in extracts of most tissues. Furthermore, the use of

fibrin assays to detect plasminogen activators which are known to augment the activity of tPA (Hoylaerts et al 1982) may result in an overestimate of fibrinolytic activity in certain tumours or an underestimate of fibrinolytic activity in a primarily uPA producing tumour when compared to normal tissue. Recently, for example, O'Grady et al (1985) found that the total plasminogen activator in breast tumours did not significantly differ from normal breast tissue, but uPA was significantly higher in malignant tissue. Similarly, Markus et al (1980) found, using a non-fibrin assay, a 2.5-4.3 fold increase in plasminogen activator in homogenates of lung tumours compared with adjacent normal tissue. In this case the plasminogen activator was mostly in the uPA form in tumour samples while tPA was the dominant species in normal tissue. Evers et al (1982) and Camiolo et al (1984) also reported 2-4 fold increase in homogenates of breast and prostate tumour tissue compared with normal adjacent tissue using an azocasein assay. At least 80% of the plasminogen activator in the tumours was in the uPA form. Findings such as these emphasize the need to identify the types of plasminogen activators present within the tissue and the use of appropriate assay procedures.

Further evidence that fibrinolytic activity in tumours is involved in tumour growth and invasion comes from in vitro studies. Increased plasminogen activator activity is an early and concomitant event in the transformation of many cell lines (Unkeless et al 1973, 1974; Ossowski et al 1973; Goldberg et al 1974; Balduzzi and Murphy, 1975; Goldfarb

and Quigley, 1978; Miskin et al 1978; Ramshaw et al 1986). In these cases this activator was determined by its Mr to be uPA (Granelli-Piperno and Reich, 1978, Dano and Reich, 1978, Dano et al 1980).

Such increases in fibrinolytic activity may account for a number of cellular alterations associated with neoplastic transformation. Changes in morphology develop with the onset of plasminogen activator synthesis (Ossowski et al 1973). Quigley et al (1979) found that cell clustering and formation of dense cellular aggregates by phorbol ester transformed cells was the result of plasminogen activator and not of plasmin although no other substrates for plasminogen activator were at that time known. More recently Keski-Oja and Vaheri (1982) detected a protein (Mr≃66,000) of the cellular matrix which seems to be a plasmin independent cellular target for urokinase. Later Del Rossi et al (1985) isolated receptors for urokinase on the cell surface of normal and transformed cells, although a function for the PA-receptor complex was not suggested.

Other studies have failed to show a consistent correlation between plasminogen activator activity and transformation (Mott et al 1974; Wolf and Goldberg, 1978; Hinuma et al 1985). However, the failure at the time to identify the types of plasminogen activator in their experimental systems and the failure to appreciate the presence of PA-inhibitors or pro-enzymes may require a re-investigation of these data.

Despite equivocal results, additional evidence from studies on tumorigenicity and metastatic potential of transformed cells further implicates plasminogen activators in tumour biology. Pollack et al (1975), Laug et al (1975) and Siskin et al (1980) all reported good correlation between the acquisition of plasminogen activator activity and the growth of neoplastic cells in semi-solid media or tumour formation in immuno-compromised animals. Ossowski et al (1980) also found that Hep-3 cells which were tumorigenic and metastasized in chick embryos released greater quantities of plasminogen activator than a subline which had lost its tumorigenicity. In other studies the correlation was less pronounced. Jones et al (1975b) found that several clones of human osteosarcoma which had high plasminogen activator activity produced tumours in immunosuppressed hamsters while others did not. Similar results were obtained by Gallimore et al (1977) using adenovirus transformed human lines and Montesano et al (1977) using transformed epithelial liver cells lines. Again few of these studies, however, meet the requirements for reliable quantitation of plasminogen activators. Only one study to date has provided conclusive evidence of a causal role for plasminogen activator mediated tissue degradation and metastasis by a tumour (Ossowski et al 1983). The authors found that by transplanting a human uPA producing tumour line (Hep-3) onto the chorioallantoic membrane of chick embryos, that antibodies which specifically inhibited human (but not chick) uPA decreased metastasis to the lung. A series of parallel experiments, showed that

inhibition of metastasis was found to be the result of uPA activity and not other immunological mechanisms. These findings would suggest that uPA is involved in the very early stages of metastasis.

Considering the evidence which suggests that a correlation may exist between fibrinolytic activity in tissues and neoplasia, there is only one report in the literature on the occurrence and distribution of PA in malignant disorders of the oral cavity. Ljunger et al (1984) immunologically identified both uPA and tPA in association with malignant neoplasms and compared these to normal tissues. Unlike other studies of normal oral mucosa, tPA was identified only in vessels of the underlying dermis. This is surprising since plasminogen activator has been detected by others in oral epithelial tissues (Birn and Fejerskov, 1971; Southam and Moody, 1981; Southam et al 1981) Birkedal-Hansen and Taylor, 1983), and uPA has been identified in epithelium from other sources including bladder, kidney tubule epithelium, pneumocytes and mammary gland epithelium (Larrson et al 1984). Plasminogen activator activity in oral tumours was also confined to tumour vessels and not to the epithelial component. The majority of PA activity was quenched using anti-tPA antiserum, however, a small fraction of the activity was identified as uPA. Similar results were achieved by Weiss and Beller (1969), Svanberg et al (1975) and Newstead et al (1976) who found that fibrinolytic activity was mainly confined to vessels

within tumours using the fibrin overlay technique, although a few studies have demonstrated plasminogen activators within carcinomas (Peterson and Zettergen, 1975; Peterson et al 1975).

The failure to demonstrate lysis in fibrinolytic autographs may be due to the presence of inhibitors in these tissues. Hashimoto et al (1985) demonstrated the presence of a fibrinolytic inhibitor in extracts of normal human epidermis. Birkedal-Hansen and Taylor (1983) also demonstrated the presence of sufficient plasminogen activator inhibitor in supernatants from cultured rat tongue keratinocytes to completely ablate endogenous activator activity. Similarly inhibitors have been detected in culture fluids from endothelial cells (Sprengers et al 1984; Van Mourik et al 1984), hepatocytes and hepatoma lines (Sprengers et al 1985), granulosa cells (Ny et al 1985), transformed fibroblasts and human blood platelets (Schleef et al 1985). The inhibitor designated PAI-1 (to distinguish it from placental inhibitor PAI-2) inhibits both uPA and tPA, although very little is known about its physiology. Immunological data from assays of PAI-1 and PAI-2 are currently lacking.

More recently the development of specific antibodies to tPA and uPA has allowed immunocytochemical localisation of plasminogen activators in tumour tissues. In the few studies so far reported uPA has been detected in human prostatic tumours (Camiolo et al 1984), melanoma cells (Markus et al 1984), and murine Lewis lung tumours (Skriver et al 1984) and

colonic tumours (Burtin et al 1985; Khoga et al 1985). Markus et al (1983) and Khoga et al (1985) also reported uPA reactivity in human colon tumours with consistent and intense staining at the luminal edge of the tumour cells which form the glands, in desquamated cells and in transitional areas between normal and tumour epithelium, morphological transition being reflected in the appearance of staining for uPA. Tissue type PA was found only in the stroma, in particular in the endothelial linings of capillaries and veins that penetrated the stroma. In contrast, Burtin et al (1985) demonstrated plasminogen activator activity in some but not all human colonic tumours. They also found that both types of activators were present in tumour cells and that the staining at best was very weak. The production of more specific, higher affinity antibodies may resolve these issues.

Evidence, though equivocal, seems to indicate a role for plasminogen activators, particularly uPA, in tumour growth. Until now many studies have failed to identify the types of plasminogen activators present in tissues or explore the presence of plasminogen activator inhibitors in assay systems. In addition, the recent isolation of pro-enzymes of plasminogen activator (Kielberg et al 1985) has meant that a reassessment of their contribution may be important in establishing a role for plasminogen activators. The recognition that tPA activity is strongly enhanced by the presence of fibrin (Hoylaerts et al 1982) makes it important

to ensure that assay methods will detect and distinguish the types of activator present in any tissue. Methods of detecting plasminogen activators are clearly important to our understanding of their biological processes. Simple extracts of whole tumours fail to take into account the heterogeneity of the tumour population and the contribution of each cell type to the total plasminogen activator pool. To a certain extent these problems have been overcome by the use of cultured cell populations. However, the problem of selection of particular populations of cells in vitro is evident (Wilson and Dowdle, 1980), and is particularly true of established cell lines.

In addition, the synthesis of plasminogen activators is influenced by physiological concentrations of certain hormones and other biologically active compounds such as EGF (Lee and Weinstein, 1978), calcitonin (Simms et al 1981; Sudol, 1985), corticosteroids (Rifkin, 1978; Mira-y-Lopez et al 1983, Busso et al 1986), parathyroid hormone and prostaglandin (Hamilton et al 1985), interleukin-1 (Mochan et al 1986) and beta-transforming growth factor (B-TGF) (Laiho et al 1986). Autocrine secretion of some of these compounds by tumours in vitro may be a complicating factor.

Clearly the use of monoclonal antibodies for localisation of both types of plasminogen activator will help to resolve some of the problems previously encountered. The future use of immunologically based assays such as radioimmunoassays and enzyme linked immunosorbent assays, which are currently being developed, may allow better

interpretation of data without the complications of proenzymes or inhibitors.

3.1.3 Assay and Detection Methods for Plasminogen Activators

3.1.3.1 Direct and Indirect Assays using Natural Substrates

Direct methods to measure PA involve the hydrolysis of esters such as p-guanidinobenzoic acid which results in a stable covalent complex between the ester and the activator. Molar concentrations of the activator can be measured by the amount of alcohol moiety released from the ester. Available active site titrants are not, however, specific for PA and can be released by other proteases. Such methods are therefore mostly used in the analysis of purified preparations of PA. Furthermore, the amplification of the response achieved by measuring plasmin in indirect systems is lost.

Most assay methods rely on indirect quantification of the enzyme activity of plasmin, generated by conversion of the proenzyme plasminogen. This can be done using a variety of natural substrates such as casein, gelatin or fibrin.

The most widely used substrate for the quantification of PA is fibrin, and the most commonly used assay is the fibrin plate assay. Samples containing plasminogen activator placed on the fibrin film, which contains plasminogen as a contaminant, cause areas of lysis due to plasmin activity. These can be measured and compared with serial dilutions of a

standard preparation of urokinase or streptokinase. It must be noted, however, that the activation of plasminogen in the fibrin plate can result from activity of other enzymes. The assay is extremely sensitive (-10 to -16 mol uPA: Jensen and Astrup, 1983) and is particularly sensitive to tPA because activation of plasminogen by tPA is stimulated by fibrin (Hoylaerts et al 1982). Refinements of the fibrin plate assay involve labelling fibrin with either I^{125} or H^3 and measuring the release of radioactive isotopes into the culture supernatant.

In addition, assays using iodinated fibrin are capable of detecting the inactive proenzyme, pro-uPA (Larrson et al 1984) when plasmin free plasminogen is used. Trace amounts of plasmin activate proactivator without measurably influencing radioactivity released by PA (Skriver et al 1982). Assays with and without trace amounts of plasmin can therefore be run in parallel.

3.1.3.2 Direct and Indirect Assays using Synthetic Substrates

Recently developed synthetic peptides have been used to quantitate PA activity by measuring the optical density of p-nitroaniline released from the substrate as a result of catalysis by plasmin. The substrate S2251 is the most commonly used for this purpose although, like plasminogen containing fibrin, it is not monospecific. By comparison with other tripeptide substrates, however, it is more sensitive by several hundred fold to plasmin than to trypsin,

thrombin or kallikrein (Soria et al 1978). Synthetic substrates for plasminogen activator (rather than plasmin) have also been developed. In these systems, plasminogen activator hydrolyses amide and ester derivatives of arginine and lysine in different tri-peptides again derivatised with p-nitroaniline. Despite their particular usefulness in measuring activation of proenzymes, some of these substrates are not specific for plasminogen activators. Furthermore, many of these substrates are not inhibited by antibodies against activators, thus limiting their usefulness in distinguishing between uPA and tPA activity (Dano et al.1985).

3.1.3.3 Zymographic Detection of Plasminogen Activators

In this process, samples containing plasminogen activator are electrophoresed in sodium dodecyl sulphate - polyacrylamide gels (SDS-PAGE). Inactivation of the enzymes by SDS is at least partially reversible by thorough removal of SDS after electrophoresis in non ionic detergent (Granelli-Piperno and Reich, 1978). The polyacrylamide gel is then overlaid with a (plasminogen containing) fibrin/agarose film into which uPA and tPA diffuse. The electrophoretic process separates uPA and tPA and gives an estimate of their M_r. Furthermore, activators and inhibitors not covalently bound are separated electrophoretically, thus removing a factor which complicates many other assay systems. Distinction between uPA and tPA can further be enhanced by incorporation of specific antibodies into the agarose overlay

film (Dano, 1980). Immunoblotting of SDS-PAGE gels has also been useful in distinguishing one chain and two chain forms of uPA in tissue lysates (Skriver et al 1984) and as a control of specificity of immunocytochemical staining.

3.1.3.4 Radioimmunoassays and Enzyme Linked Immunosorbent Assay (ELISA)

Assay methods which rely on enzymatic activation suffer from the drawback that inhibitors of PA or plasmin may be present in the biological fluids which interfere with the assay. Immunological assays directly measure PA, providing that inhibitors are not competing with antibody binding.

Several radioimmunoassays for uPA (Huber et al 1984) and tPA (McGregor and Prowse 1983; Ureden and Blomback, 1984) have been described with sensitivities of -10 to -15 mol. A number of ELISAs are also available for human plasminogen activator. Some of these assays now employ monoclonal antibodies to uPA and tPA (Herion et al 1983; Holvoet et al 1985), the use of which avoids the disadvantage of using radioisotopes or polyclonal antisera. The ELISA for tPA does not yet distinguish between one chain and two chain forms, although the presence of inhibitors has not been found to affect the detection of tPA in this type of assay.

3.1.3.5 Immunocytochemistry

Reports of immunocytochemical localisation of tPA and uPA are just beginning to appear in the literature. The production of better and more specific antibodies to the PAs

which will detect the very low levels often found in tissues are still required. Such a technique may supercede the use of the familiar fibrinolytic autograph (Todd, 1959) for the localisation of PAs within tissues. In order to distinguish tPA from uPA using autographs, specific antibodies must be used in any case, either during a pre-incubation period or by their incorporation into the film. Immunocytochemical techniques have the other advantage that PA production can be localised to a particular cell. By double labelling it may also be possible to detect whether one cell produces both types of plasminogen activators or whether different cells produce different plasminogen activators.

3.1.3.6 Summary and Objectives

Elevated plasminogen activator secretion, particularly uPA, by tumours has been correlated in a number of studies with invasion, metastases and tumorigenicity of malignant cells. The data are, as yet, equivocal but this may be the result of inappropriate methods of detection of the different types of activator, PA-inhibitors and proenzymes of plasminogen activators in samples.

The aims of the present study were therefore as follows:

1. To determine, using fibrinolytic autography staining of sections of gingival mucosa and oral squamous cell carcinomas, whether plasminogen activators could be detected in tissues <u>in vivo</u>.

2. To immunologically identify any activators present in tissues by inclusion of antibodies to uPA and tPA into fibrinolytic autographs and by immunofluorescence staining of sections.

3. To establish using epithelium grown *in vitro* from gingival epithelium and oral squamous cell carcinomas whether plasminogen activator activity is elevated in tumour epithelium compared to normal epithelium, as shown by the fibrin plate method.

4. To investigate the use of a non-fibrin based assay, the S2251 chromogenic substrate assay, to measure plasminogen activator activity.

5. To determine using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) the types of plasminogen activators produced by cultured epithelium. uPA from human urine has been shown to contain at least two active molecular forms - one with an M_r of 55,000 and an active lower molecular weight form which is a degradation product of the $M_r \simeq 55,000$ form (Soberano *et al* 1978). Several studies have shown that cell culture fluids contain uPA like activators with M_rs of 35,000, 55,000 and 80,000 - 200,000 (Astedt *et al* 1977, Vetterlein *et al* 1979; Bobbitt *et al* 1980; Wilson *et al*, 1980). Tissue type plasminogen activator is also present in some culture supernatants and can be separated from uPA on the basis of size - $M_r \simeq 70,000$ and by immunological identification after the inclusion of specific antisera to uPA or tPA

into the zymogram detector gels.

The presence of PA-inhibitor complexes and unbound inhibitors in culture fluids will be investigated in zymograms detector gels after SDS-PAGE.

3.2 MATERIALS AND METHODS

3.2.1 Fibrinolytic Autographs

Frozen sections of fresh gingival mucosa, including sulcus epithelium (10 specimens) and frozen sections of oral squamous cell carcinoma (15 specimens) were examined by fibrinolytic autography. Cultures of normal oral epithelium (8 specimens) and epithelium from oral squamous cell carcinomas (4 specimens) were also examined *in situ* by fibrinolytic autography. Cultures were grown in Lux permanox culture dishes, which were compatible with clearing agents used in staining procedures.

Fibrin films were made by evenly mixing 2% human fibrinogen (Kabivitrum, Grade L lyophilised) in 0.15M veronal acetate buffer (pH 7.4) with thrombin (Sigma 50 NIH units ml^{-1}), 10:1 vol/vol over 5um frozen sections on slides or over cells in culture dishes. A volume of 0.02 ml of fribinogen per cm^2 was required to give films of 0.03 mm final thickness. The films were incubated for 2-3 days at $4^{o}C$ before fixation in 10% neutral buffered formalin for at least 1h. The films were stained with Azure A, dehydrated, cleared in xylene and mounted. The presence of plasminogen activator was visualised as a clear area in the stained film. Immunological identification of the type of activators produced by cells was determined by pre-incubation of the sections and cell cultures with polyclonal antibodies to uPA and tPA at a dilution of 1:100 (1 $mgml^{-1}$; Biopool, Sweden). Control fibrin films were made by using essentially

plasminogen free fibrinogen (Sigma).

Veronal acetate buffer was prepared from concentrated stock buffer containing 1.5M sodium acetate and 1.5M sodium diethyl barbiturate. Working buffer was made by mixing 25 ml of veronal buffer with 25 ml of 0.1N HCl and adjusting the final pH to 7.4. This solution was made up to a final volume of 500 ml for use.

3.2.2 Immunofluorescence Staining

Normal gingival tissue (10) and tumour specimens (15) obtained at surgery were frozen in OKT mountant (Miles Scientific) in a stream of carbon dioxide vapour. Sections 5um thick were cut in a cryostat and fixed in methanol for 10 min. Monoclonal antisera to uPA and tPA, raised in mouse (Monozyme), were diluted 1:10 to 1:50 in phosphate buffered saline and applied to the sections. Sections were incubated at room temperature for 1-2 hours in humidified boxes. In control sections non-immune serum or phosphate buffered saline replaced the primary antibody. The sections were washed three times in phosphate buffered saline and then covered with fluoresceinated rabbit anti-mouse antibody at a 1:10-1:50 dilution (Dakopatts) for thirty minutes. The sections were then washed and stained with 1% haematoxylin and after rinsing with water were mounted in buffered glycerol.

Fluorescent slides were examined with a Leitz Dialux microscope (filter 12,450-490nm) and photographs were taken

using XPI 400 ASA film in either UV light or normal illumination.

3.2.3 Culture of Normal and Malignant Oral Epithelium and Fibroblasts

Cultures of epithelium from gingival mucosa and oral squamous cell carcinomas were obtained as previously described in section 2.6.2. Chapter 2. Fibroblast cell lines from gingival mucosa and oral squamous cell carcinomas were obtained from epithelial cultures, which had become overgrown by fibroblasts. These were subjected to brief trypsinisation in 0.25% trypsin and 0.02% EDTA. The supernatants, containing predominantly fibroblasts, were harvested and diluted in an equivalent volume of GMEM containing 20% FCS and centrifuged at 200g for 5 min. The supernatant was aspirated and the cells resuspended in GMEM containing 10% FCS and plated into 60 mm diameter culture dishes. Fibroblasts were routinely subcultured in a 1:2 split ratio.

3.2.4 Estimation of Cell Density in Cultures

Prior to collection of culture supernatants and cell lysates, the number of cells in each culture dish was estimated. Outgrowths of epithelial cells, clearly visible on the culture dishes were outlined in permanent pen and the areas of these outgrowths measured using a semi-automatic image analyser (Kontron MOP AM01). Where epithelial cultures were almost confluent, the entire area of the culture dish was calculated. However, throughout cultured epithelium,

spaces occurred in and between colonies and the actual surface area of the dish covered by epithelium was then estimated using a point counting method (Dunnill, 1968). Under the microscope, the epithelial outgrowths were scored along a bilateral transect (Freshney, 1987) for the presence of epithelial cells. A running mean of the percentage of the culture dish covered by epithelium was constructed for each biopsy sample cultured. Point counting continued until the running mean was stable (800 - 4000 points). In every tenth field, the number of epithelial cells per mm^2 was counted using a 1:9 graticule. From these measurements the percentage area of the dish covered by epithelium multiplied by the number of epithelial cells per mm^2 gave an estimate of the number of epithelial cells per culture dish.

For confluent oral fibroblast cultures, the number of cells per mm^2 in every tenth field of a bilateral transect of the culture dish was determined and this number was multiplied by the entire surface area of the culture dish.

3.2.5 Collection of Serum Free Cell Culture Supernatants and Cell Lysates

Cultures of normal and malignant epithelial cells after either 2 weeks or 4 weeks growth *in vitro* were washed several times in phosphate buffered saline for 2h. Cultures were re-fed with serum free MEM supplemented with 0.5% bovine serum albumin (BSA) but containing no EGF. The cultures were then incubated at high relative humidity at 37°C in an atmosphere

of 5% carbon dioxide and 95% air for 1 to 4 days. After this period of incubation culture supernatants were collected and centrifuged at 400g for 10 min. to remove cellular debris. The supernatants were then lyophilised.

The cultures were then washed twice in phosphate buffered saline and 1 ml of 2M potassium thiocyanate (KSCN) was added to each culture dish to lyse the cells. The cultures were then left at room temperature for 30 min, the cells mechanically scraped off the culture dish and the lysate containing fragmented cells was homogenised in a Griffiths tube homogeniser. The resultant lysate was centrifuged at 400g for 10 min. to remove cellular debris and the lysates lyophilised. In certain cultures cycloheximide (10 $ugml^{-1}$) was added to the serum free culture medium during the incubation period before cell culture supernatants and lysates were collected.

Before assays for plasminogen activator were performed, in order to standardise samples after their lyophilisation, the supernatants and lysates were resuspended in distilled water in a volume equivalent to 10^6 cells ml^{-1}. Further concentration or dilution of the samples was carried out, when necessary, to ensure that areas of lysis produced on fibrin plates fell within the range of streptokinase and urokinase standards.

3.2.6 The Standard Human Fibrin Plate

Stock fibrinogen was made by dissolving 1g of human fibrinogen (Kabi, Grade L) in 100 ml of 0.015M Tris buffer pH 7.8. The solution was stored in 10 ml aliquots at -20°C.

For each fibrin plate 1 ml of stock fibrinogen was added to 9 ml of Tris buffer and 0.2 ml of 0.1 M $CaCl_2$ in a 90 mm diameter petri dish. The diluted fibrinogen was clotted using 0.2 ml of bovine thrombin (Sigma) (50 NIH units ml^{-1}) by gentle mixing. The plates were left for 20 min. at room temperature on adjustable pre-levelled tables and were either used immediately or stored at 4°C for up to 3 days. Test solutions (30 ul) were applied to the surface of the fibrin plates. One 30 ul aliquot of urokinase (Leo Labs) at a dilution of 5 units per ml was applied to every fifth plate as an internal standard. The plates were incubated for 19h at 37°C on their pre-levelled tables. Areas of lysis were visualised by the addition of one drop of 0.01% bromothymol blue dye (diluted vol/vol in absolute ethanol and 0.5 N NaOH) to the test site. Areas of lysis were measured in two perpendicular diameters. Control plates contained epsilon amino caproic acid (eACA) at a final concentration of 10^{-3}M. Calibration of fibrin plates was carried out by adding serial dilutions from 20 - 0.03 U ml^{-1} of streptokinase (Kabivitrum) and urokinase (Leo Labs) to the fibrin plates. After incubating the plates for 19h at 37°C, the area of lysis in each of the five replicates was measured in two perpendicular diameters. The calibration equation was calculated from the linear regression of log concentration of activator against the area of lysis.

3.2.7 Chromogenic Substrate Assay S2251

The substrate assay was adapted from the method of Friberger et al (1978) for microtitration plates. The assay was plasminogen dependent relying on activation of plasminogen by activators present in the test samples to generate plasmin. Plasmin then catalyses the following reaction:

Sample + plasminogen $\searrow$ plasmin

H-D-Val-Leu-Lys-pNA + H20 $\longrightarrow$ H-D-Val-Leu-Lys-OH + pNA

Using a flat bottomed 96 well microtitration plate, 50 ul of test sample was added to 50 ul of plasminogen (Flow Labs) diluted in water to a final concentration of 0.06 U ml^{-1}.

The reactants were incubated at 37°C for 10 min. After this 100 ul of chromogenic substrate (final concentration 3 mM in Tris buffer 0.05 M; pH 7.4), was added to each well and the plate was incubated at 37°C. The release of p-nitroaniline(pNA), catalysed by plasmin was measured spectrophotometrically in a Multiskan spectrophotometer at 405 nm. The reaction was monitored until the end point was reached. An internal calibration was performed in each plate. Serial dilutions of streptokinase (Sigma) and urokinase (Leo Labs), from 25U ml^{-1} to 0.02U ml^{-1}, were applied to plates and linear regression analyses performed. Possible interference with absorbance readings at 405 nm, in the S2251 chromogenic substrate assay, by phenol red containing medium, was assessed at different pHs.

3.2.8 Sodium Dodecyl Sulphate - Poly acrylamide Gel Electrophoresis (SDS-PAGE)

Unless otherwise stated all chemicals were obtained from BDH Chemicals and were Electran grade.

3.2.8.1 Preparation of slab gels

Slab gel plates were pre-cleaned overnight in chromic acid, rinsed in running water and then ethanol. The clean plates were laid on paper towels and the inner aspect of each plate swabbed thoroughly with ethanol and allowed to air dry. The glass plates were separated with 1.5 mm Teflon spacers and the assembled plates clamped with metal clips positioned over the spacer positions. The clamped plate assembly was held vertical during the pouring of the gels.

3.2.8.2 Separating gel

30 % acrylamide: 0.8% bis acrylamide	20	ml
0.75 M Tris-HCl pH 8.8	30	ml
10% SDS	0.6	ml
Distilled water	6.4	ml
N,N,N^1,N^1-Tetramethylethylenediamine(TEMED)	40	ul
1% ammonium persulphate	3.0	ml

The gels were polymerised using the TEMED-Ammonium persulphate system. All the components except TEMED were mixed thoroughly and degassed. TEMED was added and the solution gently but evenly mixed and poured into the plates.

The gel was immediately overlaid with 1% SDS and allowed to polymerise for 0.5 - 1.0 h. After the gel had polymerised the 1% SDS was poured off and the surface rinsed with stacking gel solution:

3.2.8.3 Stacking gel

30% acrylamide: 0.8% bis acrylamide	1.6	ml
1M Tris-HCl pH 6.8	2.0	ml
10% SDS	0.16	ml
Distilled water	11.4	ml
TEMED	24	ul
1% Ammonium persulphate	0.8	ml

The components were mixed and degassed as before and the stacking gel poured on top of the separating gel. Immediately, a perspex comb was fitted between the glass plates into the stacking gel which was left to polymerise for 1-2 h. The comb was then carefully removed and the sample wells created by the comb rinsed with reservoir buffer. The bottom gel spacer was removed and the plates mounted in the electrophoresis apparatus (Bio Rad Slab Gel Electrophoresis Model 220). Reservoir buffer was added to the lower reservoir and any air bubbles removed from the bottom of the gel to ensure uniform electrical contact between the buffer and the gel. The top reservoir was then filled with running buffer before loading the samples.

3.2.8.4 Running Buffer

Concentrated stock running buffer was made by mixing 60.6g Tris with 288g glycine in 2L of distilled water. Running buffer was made by diluting 200 ml of stock buffer in 1.8L of distilled water and adding 20 ml of 10% SDS.

3.2.8.5 Sample preparation

Proteins contained in cell culture supernatants and lysates were dissociated by incubation of 25 ul of test sample with 25 ul sample buffer containing 2% SDS, 8M urea, 40 mM iodoacetimide and 0.2M Tris (pH 8.0). After incubation for 30 min. at 37^{o}C, the samples were made 10% with respect to glycerol containing 0.00025% bromphenol blue tracker dye. The samples (10 ul) were loaded into the sample wells formed in the stacking gel. Where appropriate tumour supernatants were diluted 1/5 or 1/10 before loading samples. The electrophoresis system was connected to a power pack and the gels were run at 20 Ma for 3-4h until the dye front had reached the bottom of the gel. Rainbow molecular weight markers (Amersham International) with molecular weights ranging from 14,300-200,000 daltons were run concurrently in a number of gels.

3.2.8.6 Zymogram overlay

The polyacrylamide gels were washed in 1 L of 2.5% aqueous Triton-X-100 for 1h and then repeatedly rinsed with distilled water and the gels were carefully laid onto indicator gels. Fibrin agarose indicator gels were prepared

by mixing 16 ml of 1% agarose (LKB) solution boiled for 10 min and cooled to 42°C, and 4 ml of fibrinogen (2 $mgml^{-1}$) and 120 $ugml^{-1}$ of plasminogen were added. Thrombin (120 ul of 10 Uml^{-1}) was added to the solution which was poured onto 10 mm x 15 mm gel electrophoresis films and allowed to form a firm gel.

The acrylamide gel-indicator gel sandwich was placed in sealed humidified containers overnight at 37°C. Areas of lysis in the indicator gels produced by plasminogen activators were visualised as clear areas in the fibrin film. Control zymograms were prepared with plasminogen free fibrin (Sigma). Immunological identification of plasminogen activators in gels was carried out by incorporation of antibodies to uPA and tPA into the zymogram overlays. A 0.4% solution of tPA (5 $mgml^{-1}$) or uPA (5 $mgml^{-1}$) was added to each zymogram.

Additionally, fibrinolytic inhibitors could be detected in zymograms, in which the fibrin was completely lysed in the zymogram during the incubation period by the addition of urokinase at a concentration of 0.075 Uml^{-1}. Inhibitors present in samples resulted in opaque zones in the zymograms where fibrin was not lysed.

3.3 RESULTS

3.3.1 Fibrinolytic Autography

Frozen sections of human gingival mucosa showed lysis over crevice epithelium and over vessels in the connective tissue. Pre-incubation of sections with polyclonal antisera to uPA and tPA indicated that lysis over crevice epithelium was due to the presence of both urokinase and tissue type plasminogen activators (Figs. 60 and 61). More extensive lysis was observed after pre-incubation of sections with antibodies to uPA than with antibodies to tPA indicating that the majority of the lysis resulted from tPA activity. Lysis over vessels in the connective tissue was also mainly due to tissue activator (Figs. 62 and 63). No lysis was observed in sections coated with plasminogen free fibrin or in sections which had been pre-incubated with antiserum to both uPA and tPA. Lysis was therefore due to the presence of plasminogen activators and not plasmin or other proteolytic enzymes.

Lysis occurred over each of the eight cultures of normal gingival epithelium and was confined mainly to the circumference of the epithelial outgrowths (Fig. 64). Virtually all of the plasminogen activator activity in cultures was inhibited by the inclusion of antiserum to uPA into the fibrin films. Infrequently very small areas of lysis occurred over cultures coated with plasminogen free fibrin or in fibrin films in which uPA and tPA antiserum had been incorporated. This lysis may have been due to the production of plasmin or other proteolytic enzymes by

FIG. 60

Lysis in fibrinolytic autograph over gingival crevice epithelium after pre-incubation of sections with anti-urokinase antibodies (x40 mag).

FIG. 61

Lysis in fibrinolytic autograph over gingival crevice epithelium after pre-incubation of sections with anti-tPA antibodies (x40 mag).

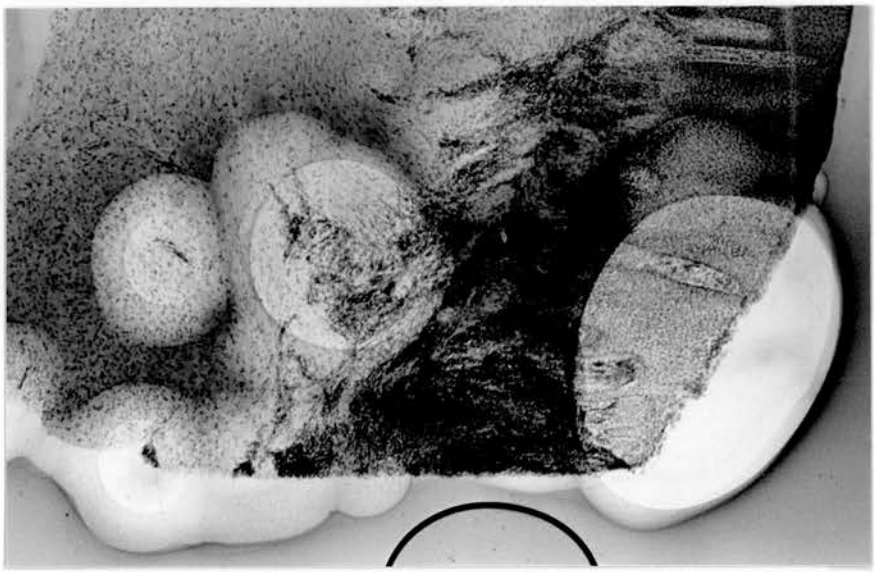
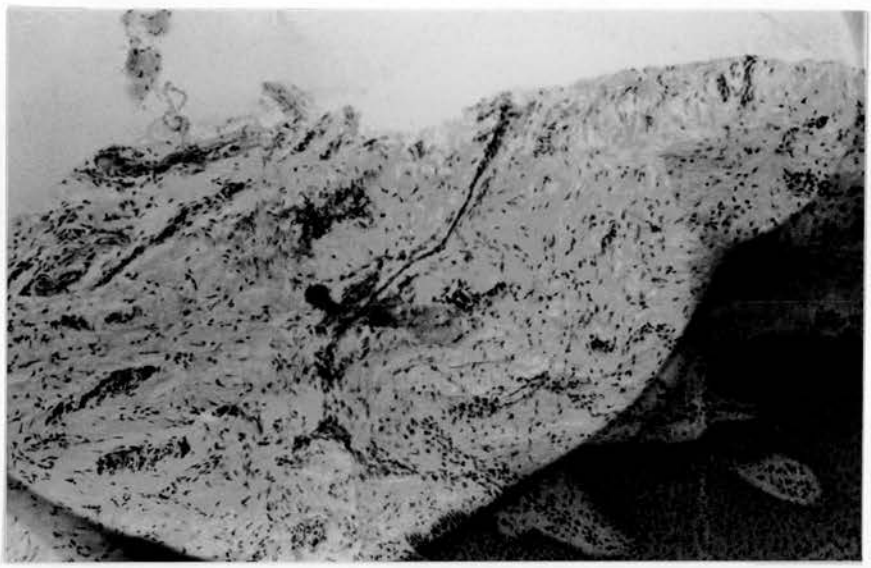


FIG. 62

Lysis in fibrinolytic autographs of gingival mucosa occurring over vessels. Pre-incubation with anti-uPA antibodies does not markedly inhibit lysis (x40 mag).

FIG. 63

Lysis in fibrinolytic autographs of gingival mucosa occurring over vessels. Sections pre-incubated with anti-tPA antibodies do not inhibit all lytic activity (x40 mag).



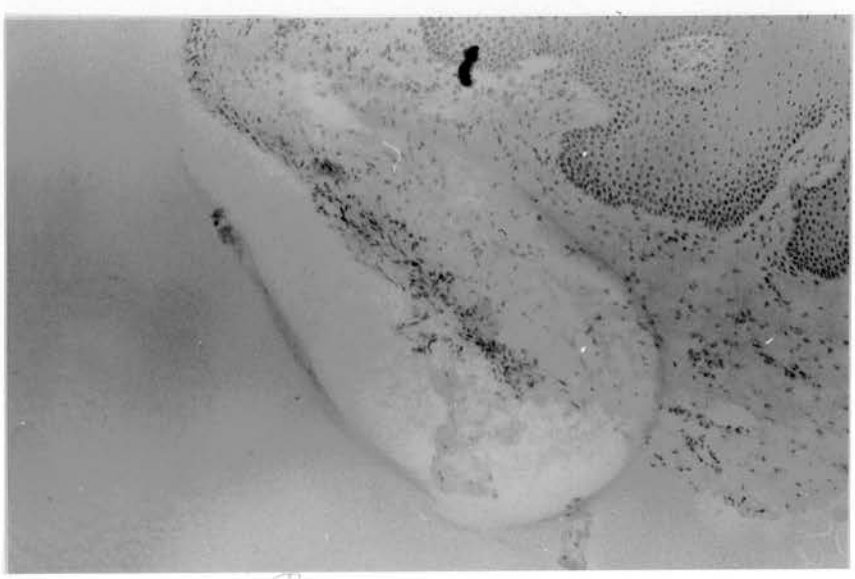
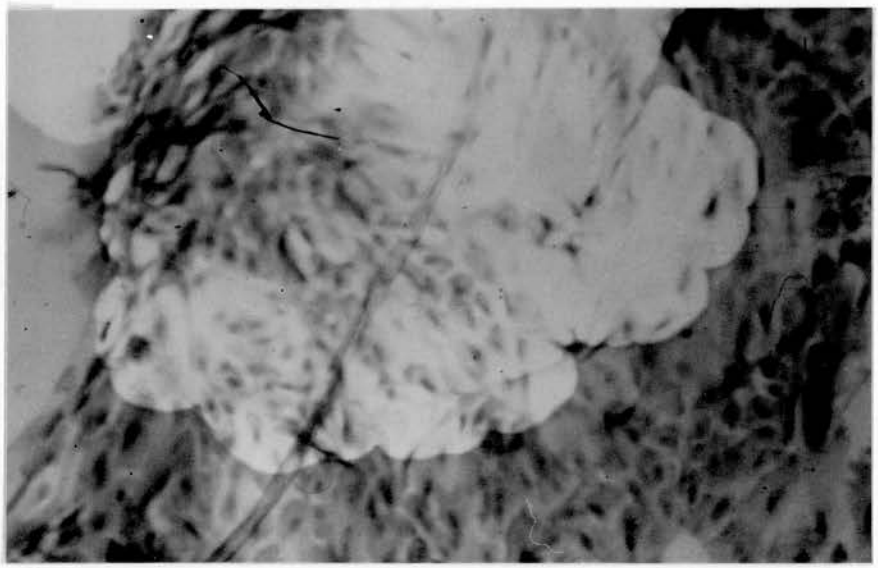


FIG. 64

Fibrinolytic autograph of normal cultured gingival epithelial cells. Lysis occurs mainly at the migrating edge of epithelial colonies (x200 mag).



inflammatory cells present in the cultures (see Chapter 2). In addition plasmin may have been converted from trace amounts of plasminogen bound to cells, previously incubated in serum containing medium. This activity was, however, very low and was only evident after prolonged incubation of the cultures (i.e. 4 or more days at $4^{o}C$).

In frozen sections of oral squamous cell carcinomas no lysis was observed over tumour epithelial cells (Fig. 65). Lysis in these sections, where present, was confined to endothelial cells in vessels and the major part of the fibrinolytic activity was quenched by pre-incubation of sections with antiserum to tPA. No lysis was observed over sections which were coated with plasminogen free fibrin or after pre-incubation of sections with antiserum to uPA and tPA, indicating that lysis was due to plasminogen activators.

Lysis was observed over one or two epithelial cells in only one epithelial outgrowth established from a biopsy of an oral squamous cell carcinoma (Fig. 66). In the remaining three biopsies of oral squamous cell carcinomas, no fibrinolytic activity was observed in 36 of the epithelial outgrowths from explants.

3.3.2 Immunofluorescence Staining

The results of staining of frozen sections of gingival mucosa and of oral squamous cell carcinomas with different concentrations of antibody to uPA and tPA are shown in Table 17. Maximum intensity of staining was achieved with

FIG. 65

Fibrinolytic autographs of section through oral squamous cell carcinoma (x200 mag).

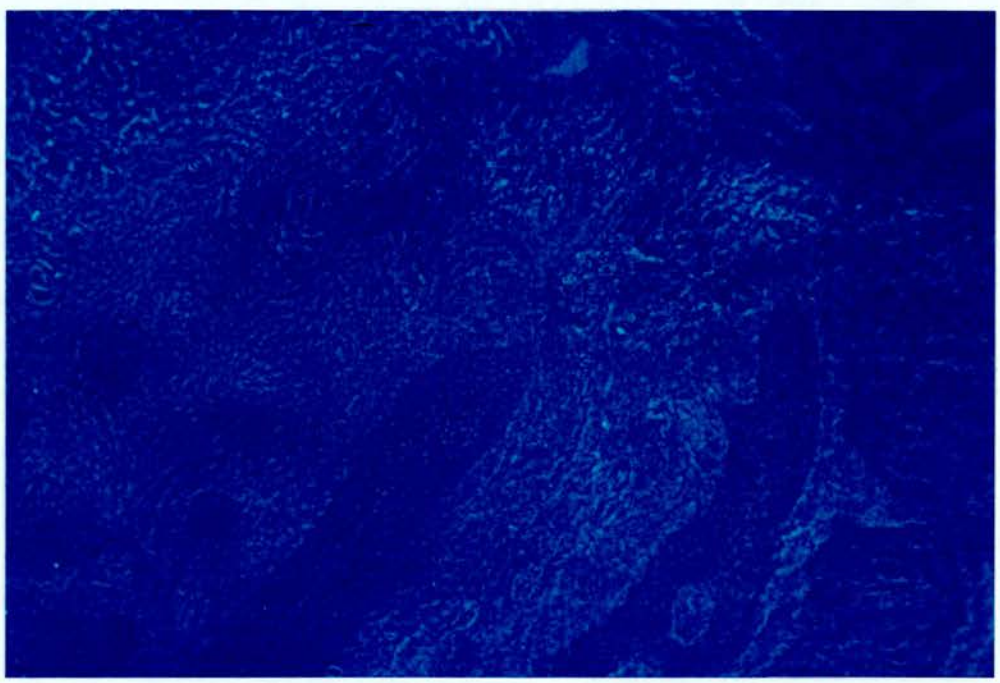


FIG. 66

Lysis in fibrinolytic autograph of cultured oral squamous cell carcinoma.

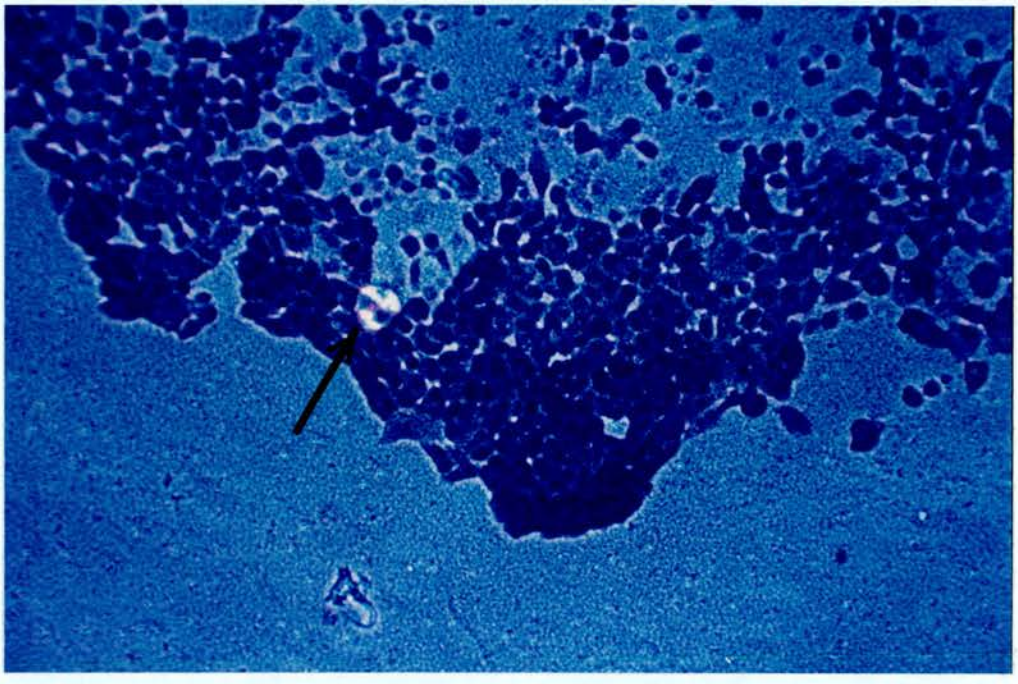


TABLE 17

OPTIMISATION OF INDIRECT IMMUNOFLUORESCENCE STAINING WITH tPA AND uPA ANTIBODIES

Primary Antibody	Dilution	Dilution of FITC Conjugated Antibodies	Staining Characteristics	Intensity
Anti uPA and anti tPA antibody	1:10	1:10 1:20 1:50	Fluorescence observed in cells in sections with no background staining Weak fluorescence observed in cells in sections	+++ ++
Anti uPA and anti tPA antibody	1:20	1:10 1:20 1:50	Fluorescence observed in some sections with little background staining Weak staining observed in some sections	++ ++
Anti uPA and anti tPA antibody	1:50	1:10 1:20 1:50	No staining apparent	-
Non Immune serum	1:10	1:10	Background staining sometimes observed only in dead cells in sections	-
PBSA	-	1:10	No background staining observed	-

dilutions of 1:10 to 1:20 of the primary antibody and a 1:10 dilution of the secondary antiserum. Staining became progressively weaker with lower dilutions of primary antibodies and no staining was observed with dilutions below 1:50. No background staining was observed in the absence of primary antibody or in sections treated with non-immune serum.

In gingival mucosa, immunofluorescence was observed in vessels stained with antiserum to uPA and tPA. No fluorescence was observed in gingival epithelium in immunostained sections.

In the specimens of tumour sections examined in this way a number showed immunofluorescence in endothelium and connective tissue after staining with antiserum to uPA (Fig. 67) and in endothelial cells lining vessels after staining with tPA and uPA antiserum (Fig. 68). In sections from four of the fifteen specimens, fluorescence was observed in isolated tumour epithelial cells after staining with antiserum to uPA or tPA but staining was always very weak (Fig. 69-72). No fluorescence was observed in sections in which primary antiserum was replaced with phosphate buffered saline or non immune serum.

3.3.3 In Vitro Assays for Plasminogen Activator

3.3.3.1 Estimation of cell number in vitro

An estimate of cell numbers was made for all cultures used in the experiments described in this chapter. Figure

FIG. 67

Immunofluorescence in connective tissue in a section of an oral squamous cell carcinoma after staining with antibodies to uPA (x200 mag).

FIG. 68

Immunofluorescence in vessel endothelium in a section of an oral sqaumous cell carcinoma after staining with antibodies to tPA (x200 mag).

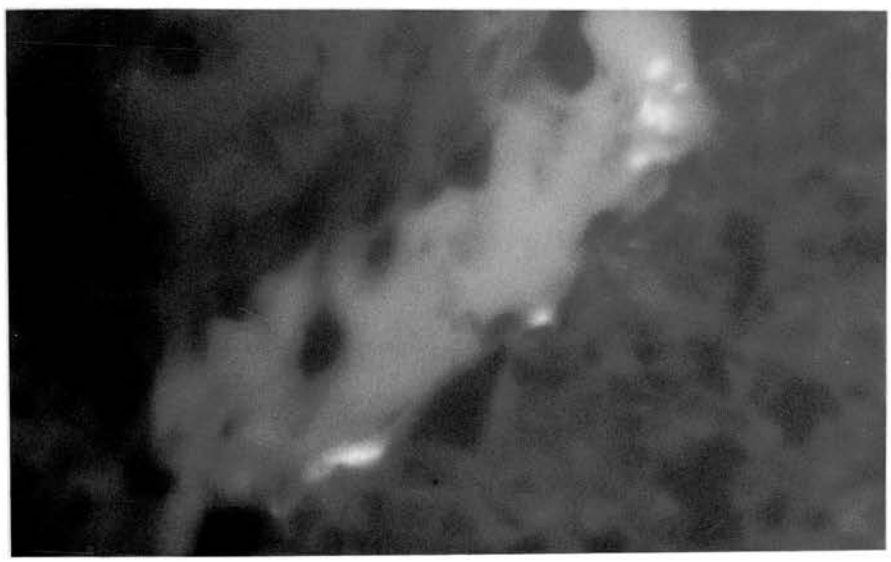
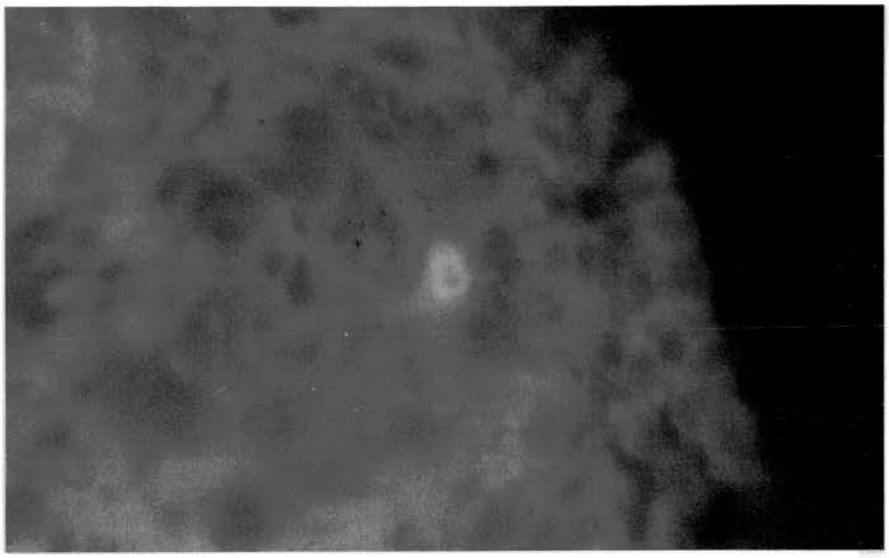


FIG. 69

Section of oral squamous cell carcinomas stained by indirect immunofluorescence with antibodies to uPA (x200 mag).

FIG. 70

Light micrograph of Fig. 69



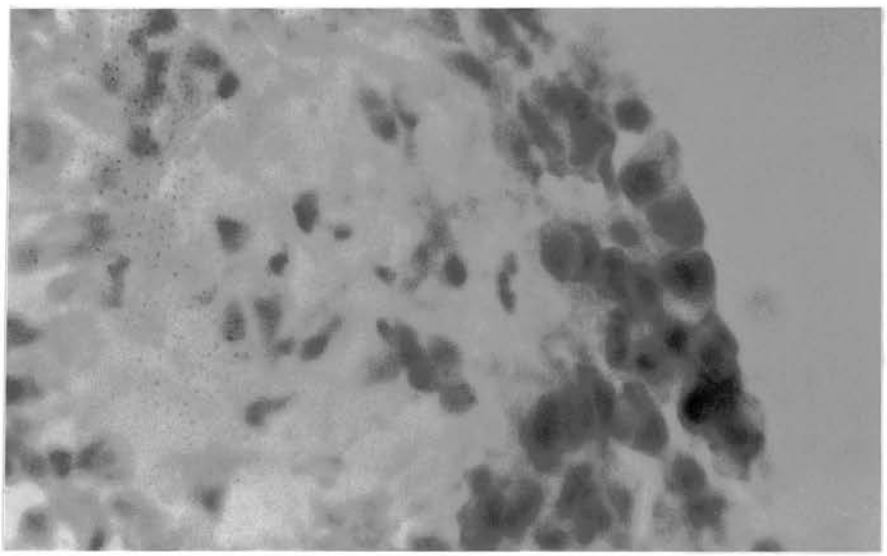
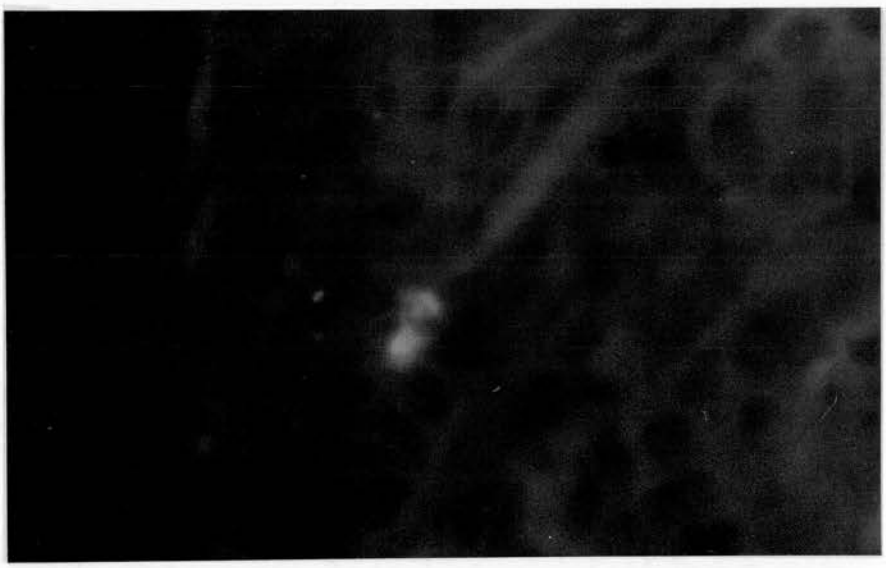


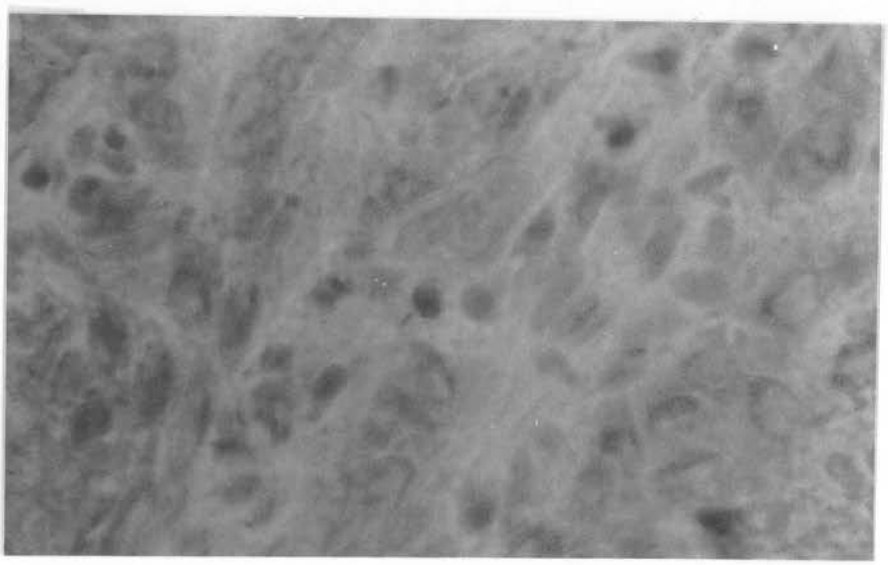
FIG. 71

Section of oral squamous cell carcinoma stained by indirect immunofluorescence with antibodies to tPA (x200 mag).

FIG. 72

Light micrograph of Fig. 70





73 shows an example of results obtained for cultures from one patient biopsy. In this example over 1700 points were counted in order to demonstrate a stable mean percentage area of the culture dishes covered by epithelium. The range of points which had to be counted before a stable mean was obtained for each series of patient cultures ranged between 800-4000 points (see Table 11).

3.3.3.2 Calibration analyses of the fibrin plate assay

A linear regression analysis of log concentration of activator (as x) against area of lysis (as y) was performed for streptokinase and urokinase (Figs. 74 and 75). The calibration equation was linear across the range of concentrations for streptokinase (r^2 = 0.96). Lysis of substrate by urokinase was only linear in the range 1.25 Uml^{-1} - 25U ml^{-1} (r^2 = 0.85). Below 1.25U ml^{-1}, no discrimination could be made between plaminogen activator activity and background lysis caused by buffer.

These calibration equations were used throughout this study. In each experiment urokinase and streptokinase (5 Uml^{-1}) were added to every fifth plate to ensure reproducibility of the assay. When the lyophilised samples from cultured epithelium were resuspended, the concentration of plasminogen activator in the samples was adjusted, where necessary, to ensure their assay within the established linear part of the scale. The fibrin plate was more sensitive to streptokinase than urokinase and streptokinase

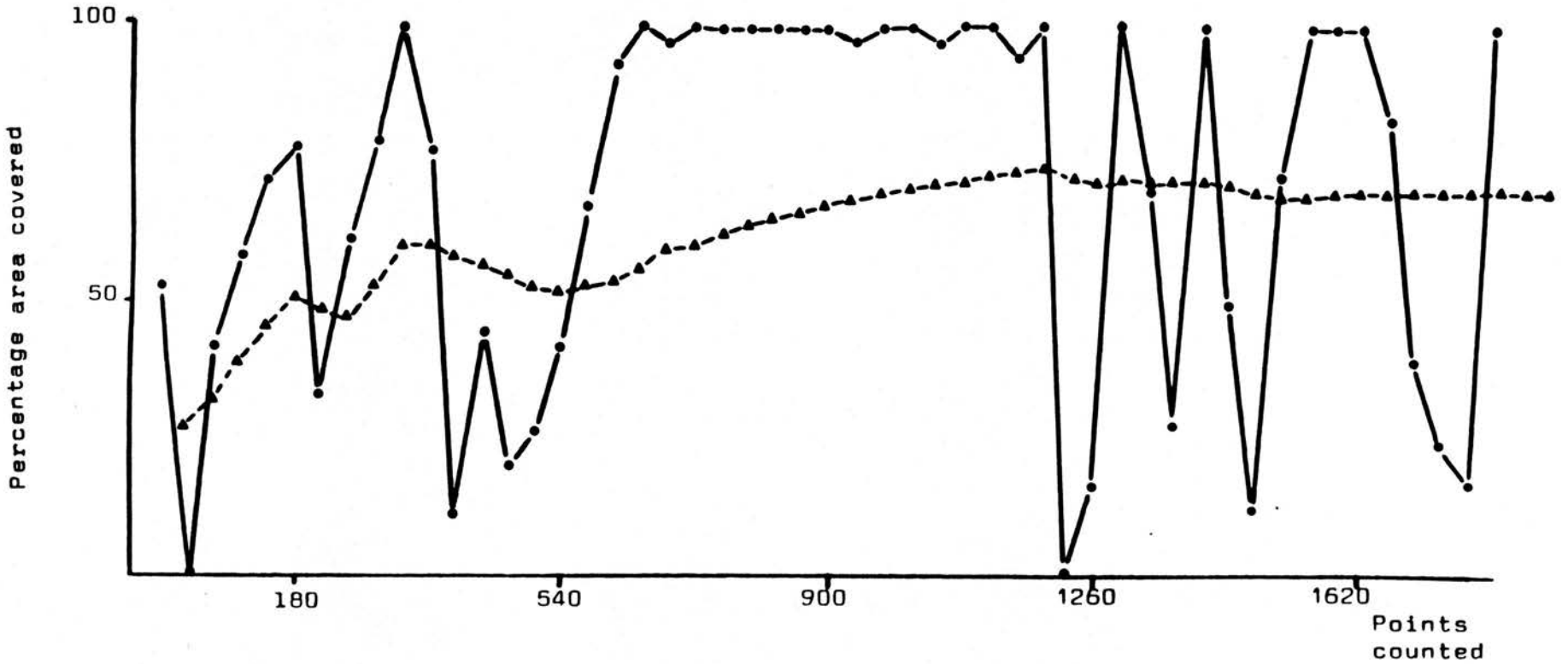


FIG. 73
Estimation of percentage area of culture dish covered by epithelium. 36 points were counted in a single field (●—●) and a running mean (▲—▲) was calculated.

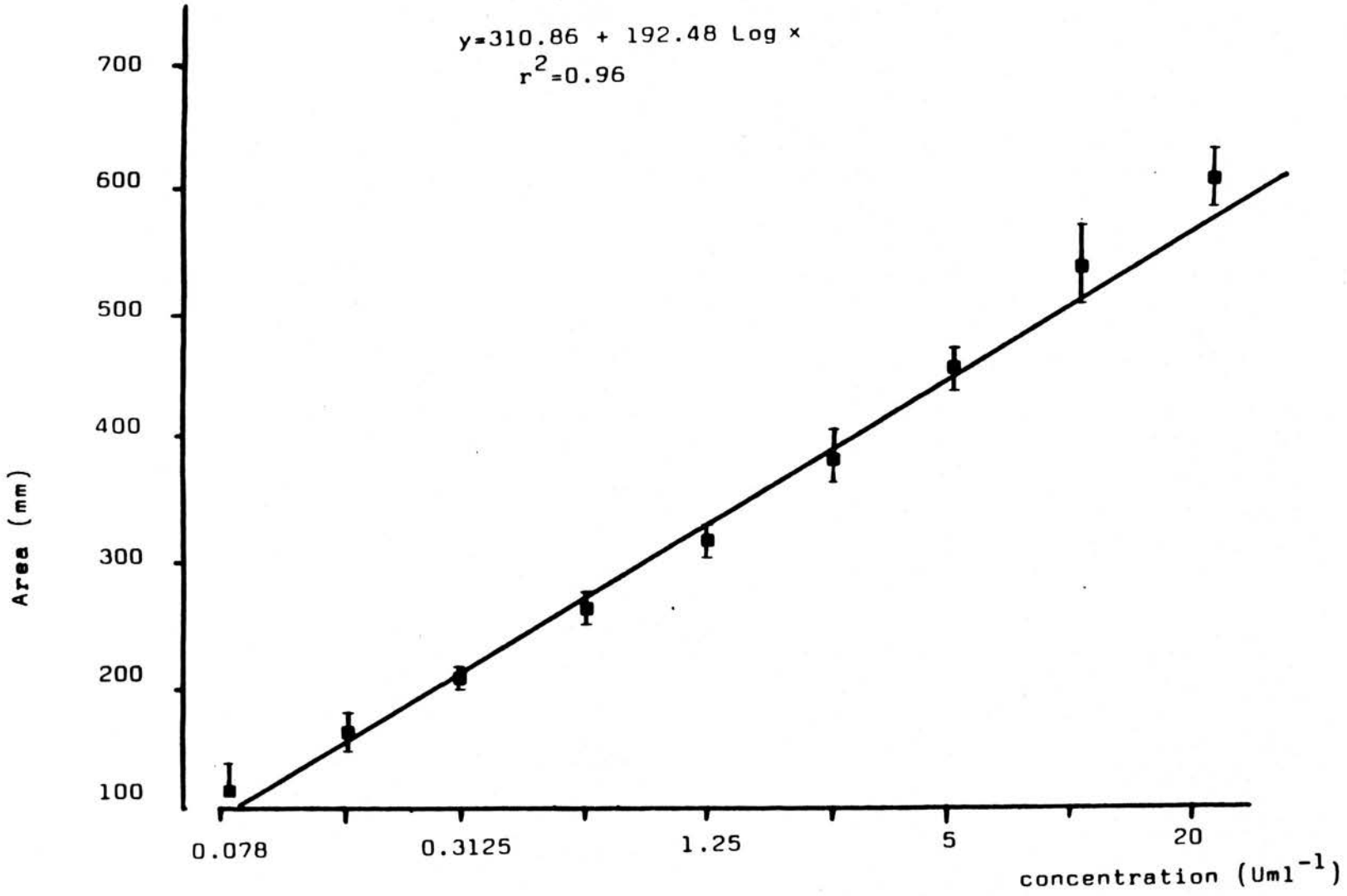


Fig.74 **Calibration equation for activation of plasminogen by streptokinase in fibrin plate analyses.** (±SD, n=5)

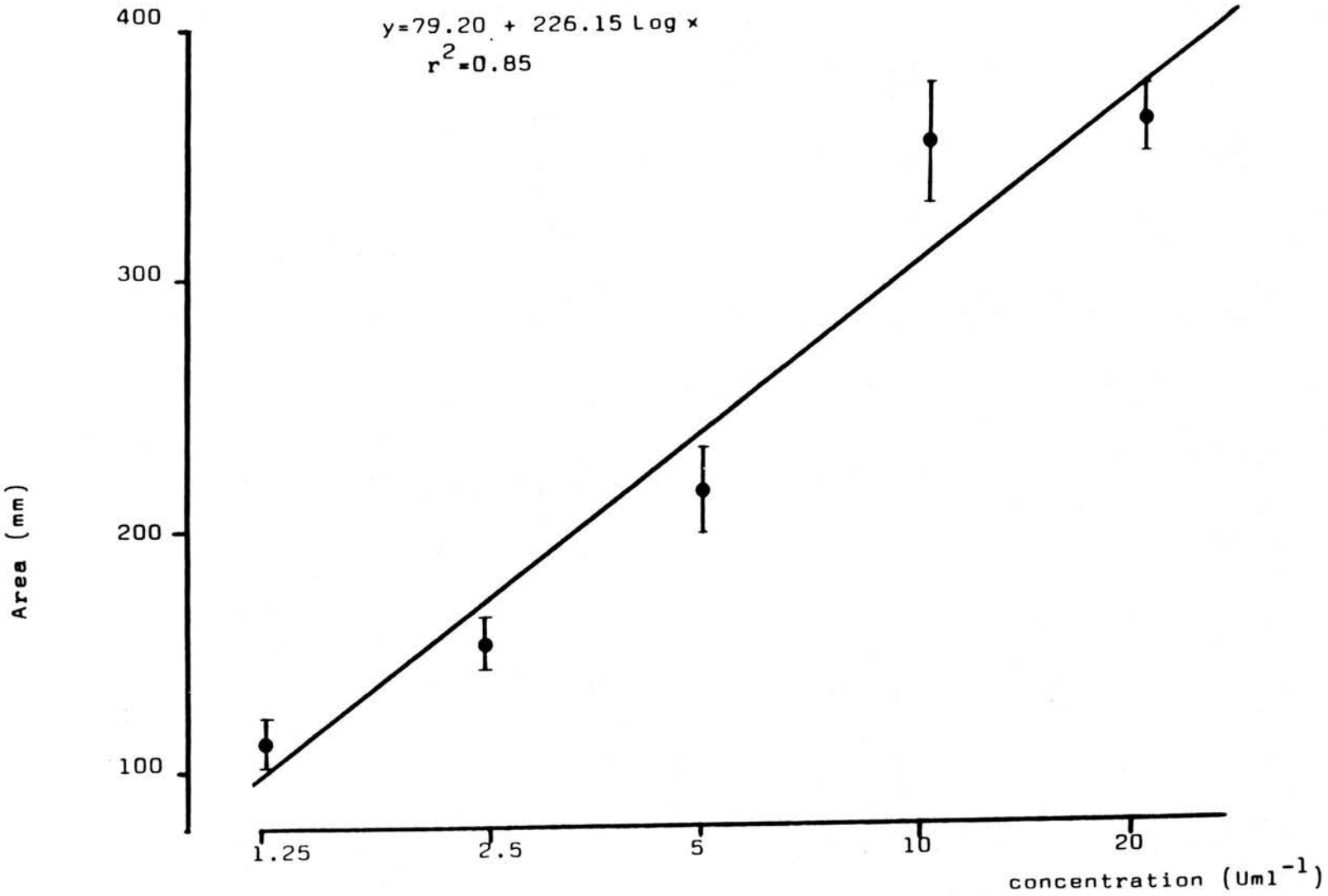


Fig.75 Calibration equation of plasminogen by urokinase in fibrin plate analyses. (±SD, n=5)

was therefore used as a standard reference in subsequent experiments using the fibrin plate assay.

3.3.3.3 Calibration and characterisation of the chromogenic substrate assay

Linear regression analyses of the log concentration of activator against absorbance (Fig. 76) indicated that good linearity was observed in the concentration range 0.19 -25.0U ml^{-1} for both streptokinase (r^2= 0.98) and urokinase (r^2 = 0.91). Using lower dilutions of activators 0.019U ml^{-1} to 2.5U ml^{-1} activation of substrate by streptokinase and urokinase reached a plateau (Fig. 77). Sensitivity of this assay was therefore similar to the fibrin plate method.

Possible interference in absorbance readings at 405 nm in the S2251 assay by phenol red contained in the medium was then assessed. If phenol red was present, the optical density was slightly reduced at pH 7.0 across the range of concentrations of streptokinase used (Fig. 78). This reduction could, however, be minimised if the background reading was subtracted at the beginning of the assay. At pH 7.4, however, the colour of the supernatants altered from orange/red to bluish/red and the absorbance readings at 405 nm were markedly altered (Fig. 78). Using concentrations of streptokinase between 1.5 - 25U ml^{-1}, considerable quenching of absorbance occurred (Fig. 78). With lower dilutions of streptokinase an increase in absorbance, compared to the non phenol red containing control was observed. This assay was therefore not further used in this study.

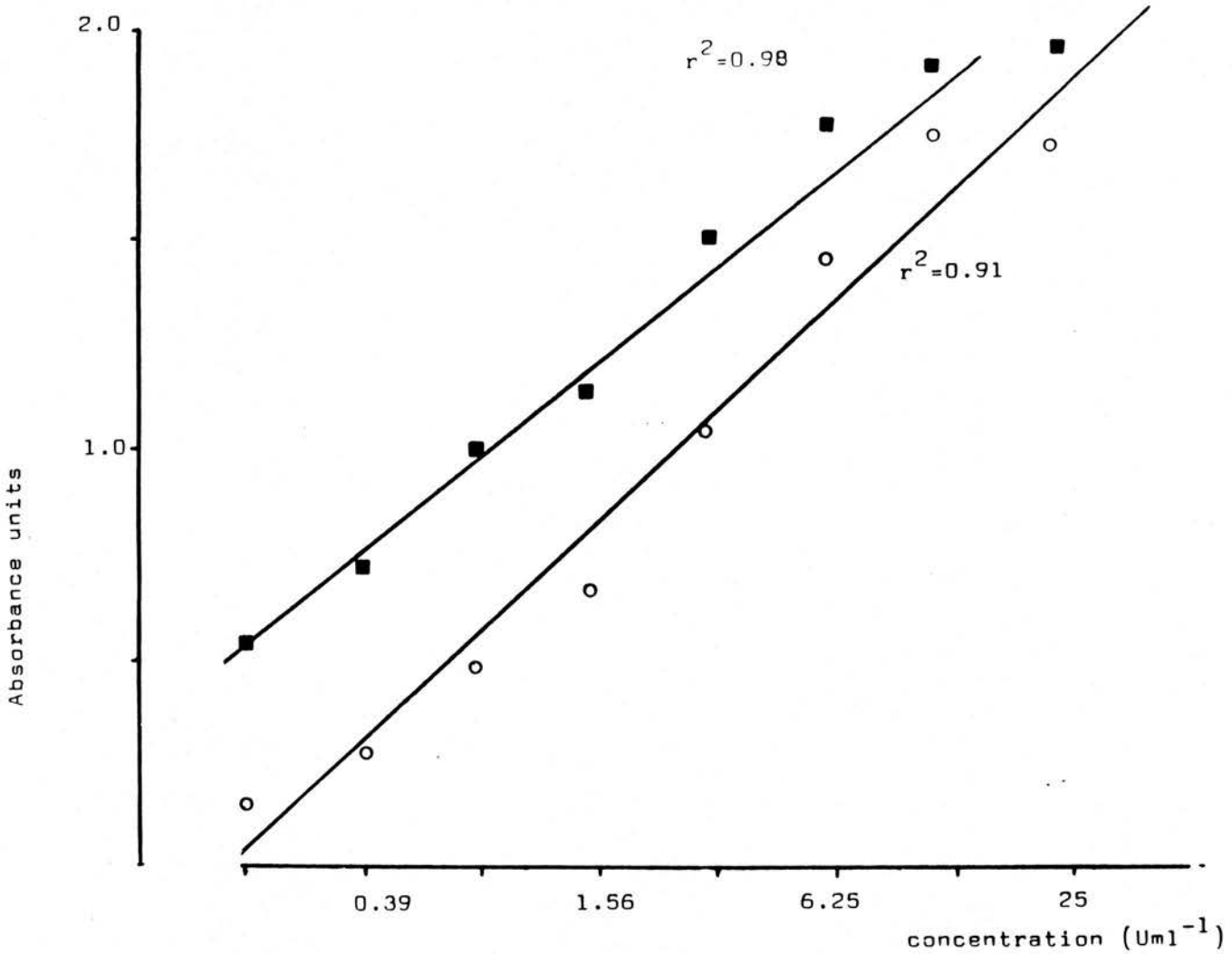


Fig.76 Activation of S2251 substrate by streptokinase (■—■) and urokinase (o—o) n=3

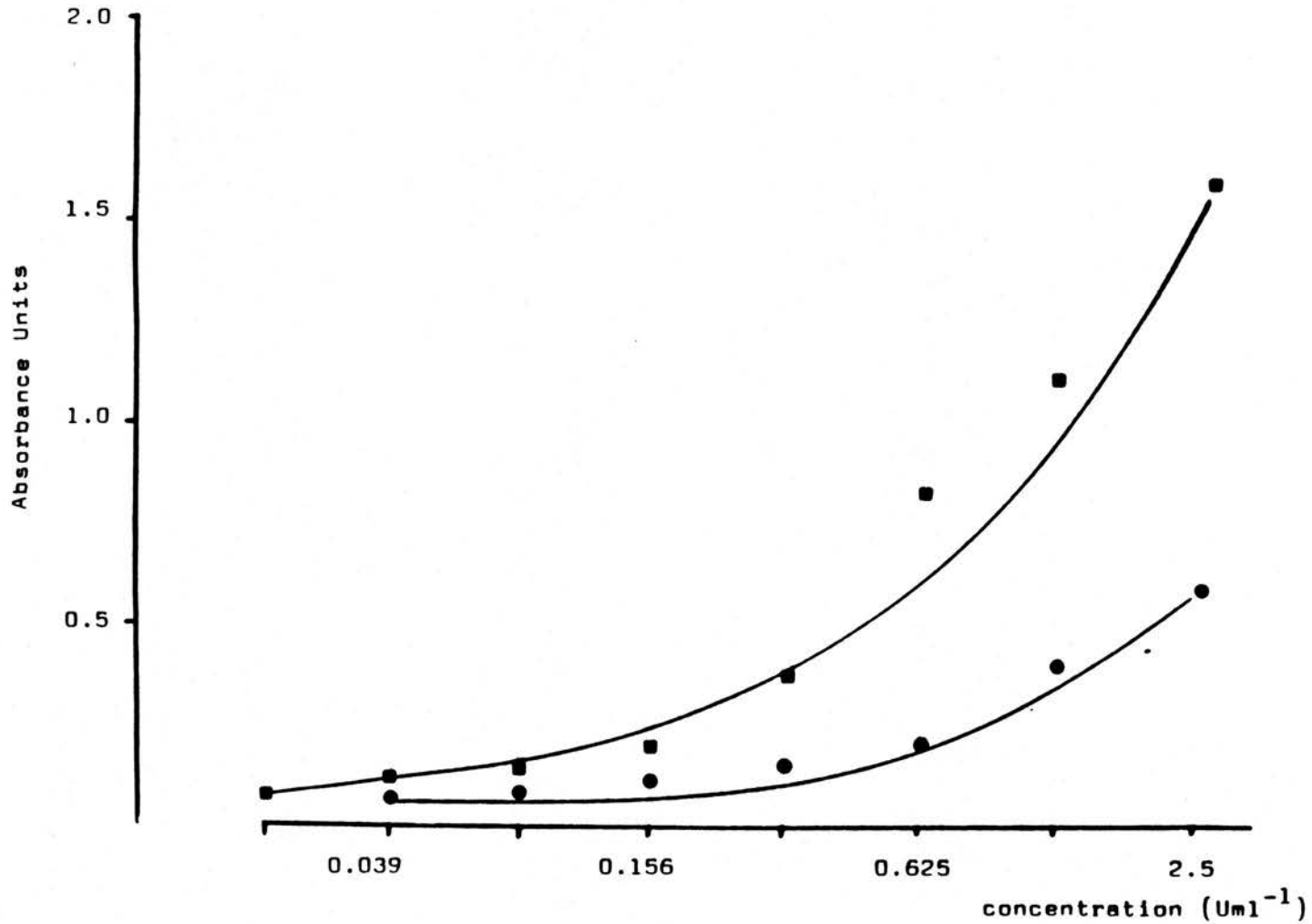


Fig.77 Release of p-nitroaniline from chromogenic substrate S2251 after activation by low concentrations of streptokinase (■—■) and urokinase (●—●) n=3

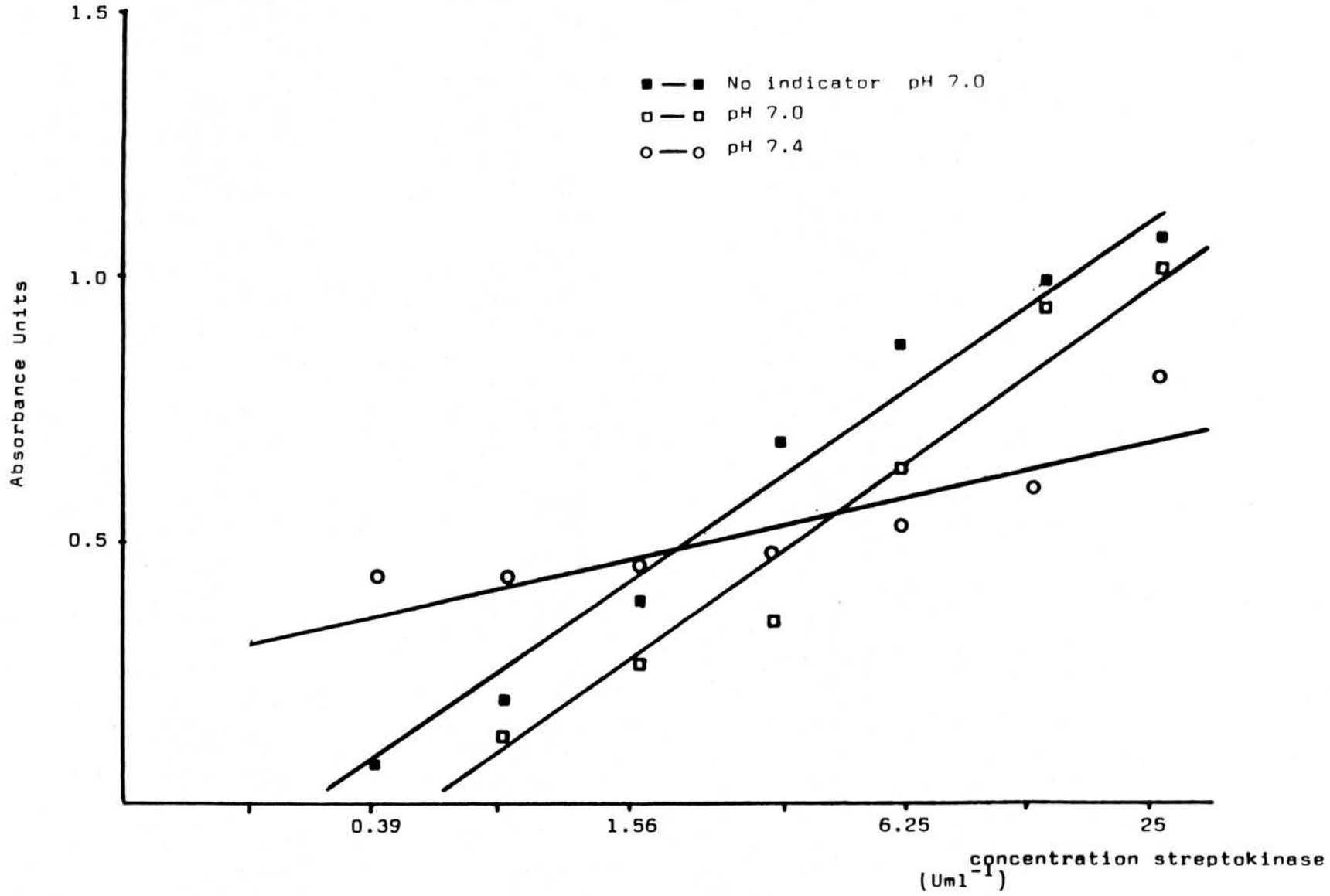


Fig.78 **The effects of phenol red indicator on absorbance in the chromogenic substrate assay.** n=4

3.3.4. Fibrin Plate Analysis of Oral Keratinocyte Culture Fluids

Cell lysates collected from two week old cultures of normal gingival epithelium showed little or no cell associated activity (Table 18 Fig. 79). Levels of plasminogen activator secreted into the medium were higher with a mean value of $5.1U/10^7$ cells after one day in serum free medium. Variation between samples was considerable with concentrations of activator ranging from 0.5 - $23U/10^7$ cells.

After two days in serum free medium more cultures expressed cell associated activity (Table 19 Fig. 79) but overall activity remained low ($<1U/10^7$ cells). Plasminogen activator activity in supernatants, however, increased substantially over the two day period from a mean value of $5.1U/10^7$ cells to $28.2U/10^7$ cells. Inter-patient variation persisted in supernatants harvested after two days with plasminogen activator activities ranging from 0-236.2 $U/10^7$ cells.

In gingival epithelium cultured for four weeks *in vitro*, cell associated plasminogen activator activity was only slightly increased when compared to keratinocytes grown for two weeks *in vitro* (Table 20 Fig. 80). Plasminogen activator activity in supernatants, however, increased from less than $2U/10^7$ cells after one day in serum free medium to a mean value of $244.6U/10^7$ cells after two days in serum free medium. Marked variation in activities in supernatants was

TABLE 18

FIBRIN PLATE ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL ORAL KERATINOCYTES AFTER 2 WEEKS IN VITRO. SAMPLES WERE HARVESTED AFTER 1 DAY IN SERUM FREE CONDITIONS

Sample N°	Activity (Units/10^7 cells)	
	Lysates	Supernatants
1	0	3.1
2	0	0.5
3	0.6	2.9
4	0	23.0
5	3.6	8.6
6	0	4.1
7	0.2	2.1
8	0	0.4
9	0.8	1.0
10	0	0.4

TABLE 19

FIBRIN PLATE ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL ORAL KERATINOCYTES AFTER 2 WEEKS IN VITRO. SAMPLES WERE HARVESTED AFTER 2 DAYS IN SERUM FREE CONDITIONS

Sample N°	Activity (Units/10^7 cells)	
	Lysates	Supernatants
1	0.2	4.4
2	0.3	5.1
3	0	0.5
4	0.3	0.8
5	0	7.7
6	0	0.5
7	0.2	0
8	0.9	236.2
9	0.1	47.5
10	1.1	36.9
11	0.3	15.7
12	0.5	5.3
13	0.3	7.1

TABLE 20

FIBRIN PLATE ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL KERATINOCYTES AFTER 4 WEEKS IN VITRO. SAMPLES WERE HARVESTED AFTER 1, 2 AND 4 DAYS IN SERUM FREE CONDITIONS

Day Harvested	Sample No.	Activity (Units/10^7 cells)	
		Lysates	Supernatants
1 day	1	0.7	2.1
	2	1.1	1.6
	3	1.2	0.7
	4	0.1	0.9
	5	0.7	3.1
2 days	1	1.6	3.0
	2	1.9	17.1
	3	0	47.5
	4	3.6	14.6
	5	0.2	372.4
	6	0.6	47.0
	7	0.2	1427.0
	8	0	8.0
	9	0.9	264.4
4 days	1	0.21	216.3
	2	0.40	49.1

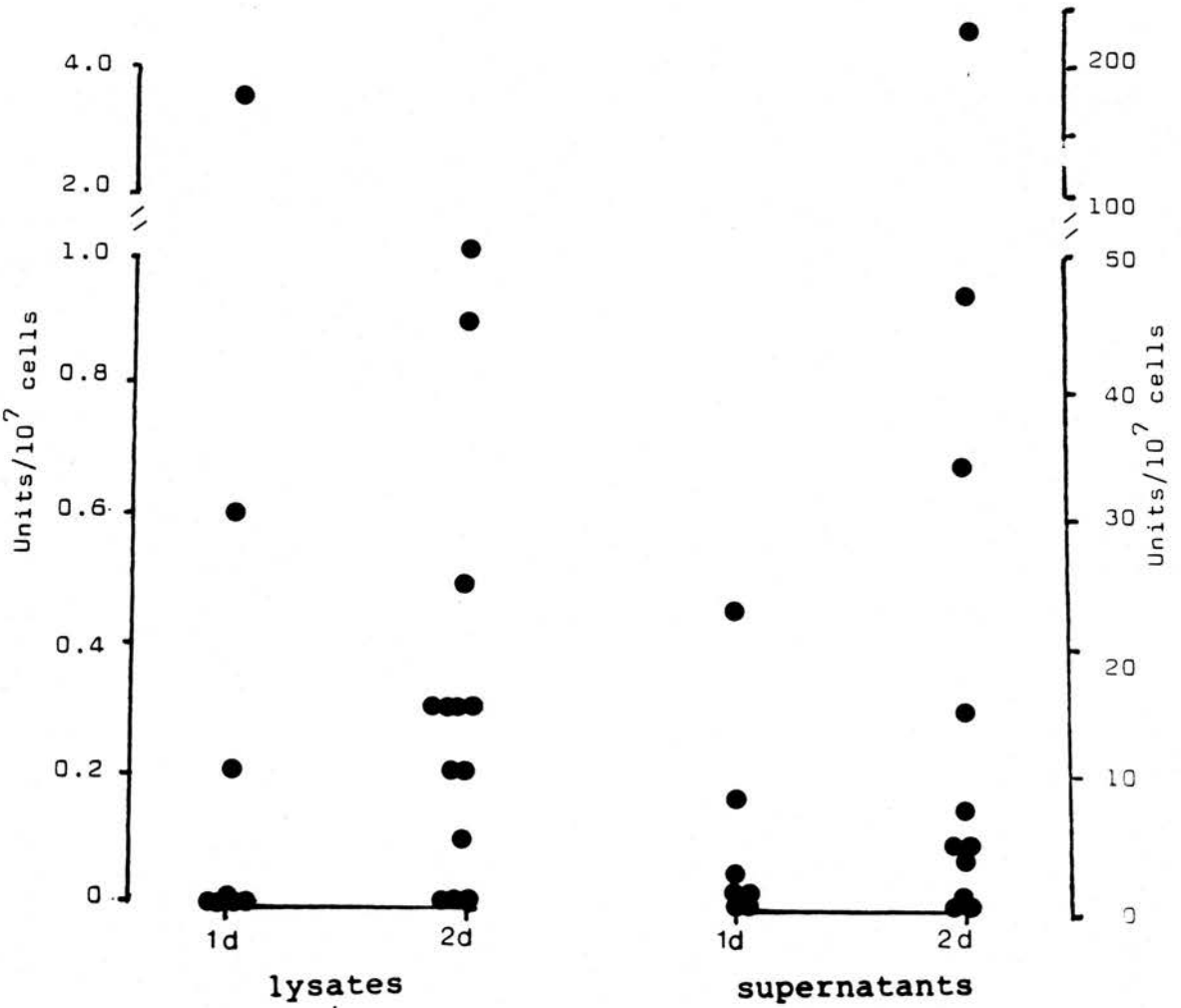


Fig.79 **Plasminogen activator activity in cell lysates and culture supernatants from normal oral keratinocytes grown for two weeks in vitro.**

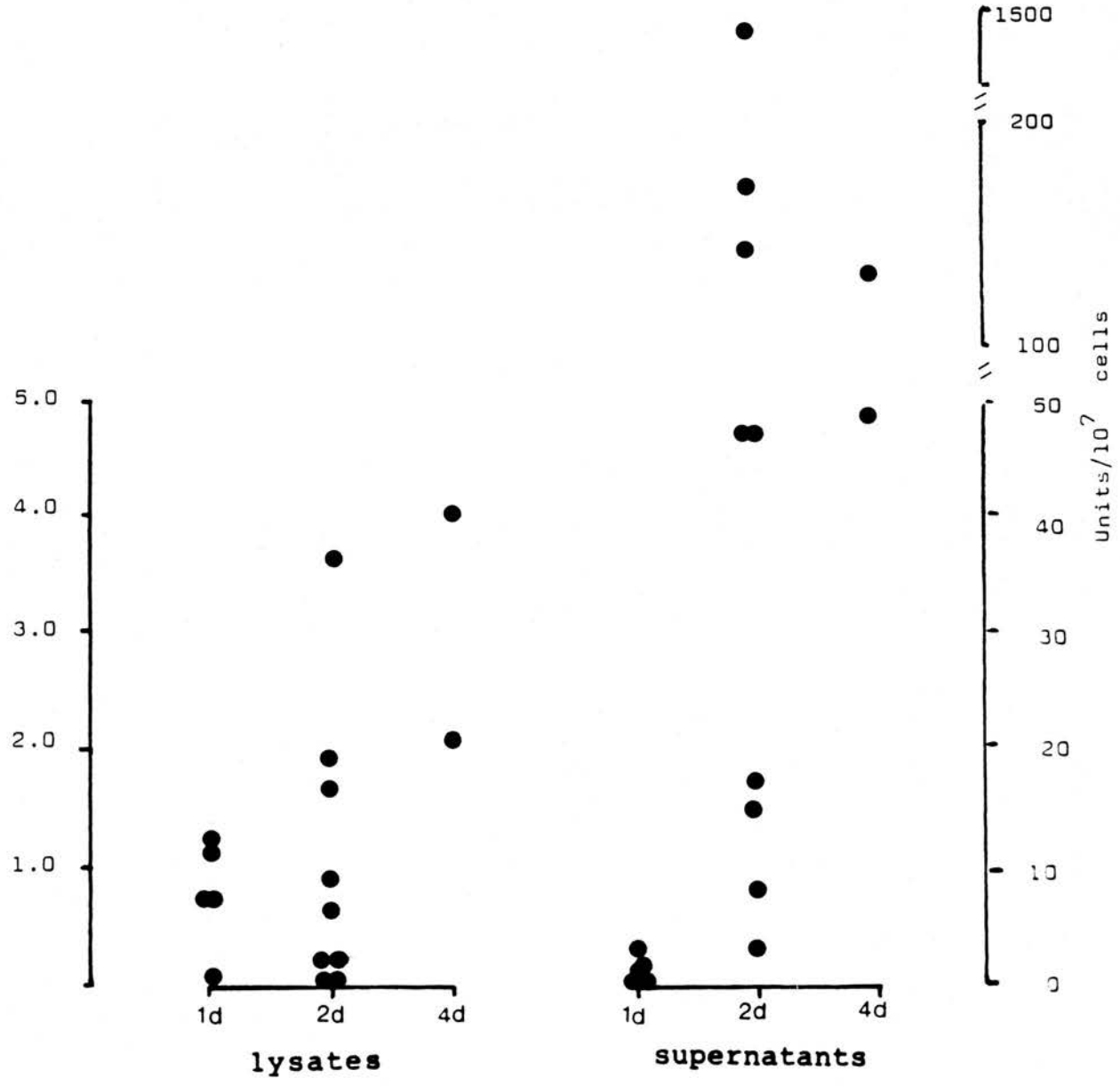


Fig.80 Plasminogen activator activity in cell lysates and culture supernatants from normal oral keratinocytes cultured for four weeks in vitro.

still apparent in four week old cultures ranging from 3.0U/10^7 cells to 1427U/10^7 cells. Despite the ten fold increase in plasminogen activator in four week cultures (244.6U/10^7 cells) compared to those found in two week old cultures 28.2U/10^7 cells) these differences were not statistically significant (Mann-Whitney U Test p=0.1), and probably results from the large inter-patient variation in samples. Accumulation of plasminogen activator in supernatants from four week old cultures was also examined after four days in serum free medium. After four days cells in serum free culture conditions appeared granular, accumulated intracytoplasmic vacuoles and many cells contained pyknotic nuclei. Data for only two samples are therefore shown (Table 20, Fig. 80) and these fell within the range of plasminogen activator activity found in supernatants harvested after two days. No further data for this time point were collected. On control fibrin plates containing 10^{-3}M epsilon amino caproic acid, which inhibits plasminogen activator activity, no lysis was observed on plates after incubation with any of the samples. Furthermore, no plasminogen activator activity was detected in culture supernatants after cultures were incubated during the two day period with the protein synthesis inhibitor cycloheximide indicating that plasminogen activator present in samples was synthesised de novo during the sample collection period.

Plasminogen activator activity in tumour cultures grown for four weeks in vitro was measured after two days in serum free medium. The data in Figure 81 and Tables 21 and 22

TABLE 21

FIBRIN PLATE ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY OF ORAL SQUAMOUS CELL CARCINOMAS AFTER 4 WEEKS IN VITRO. SAMPLES WERE HARVESTED AFTER 2 DAYS IN SERUM FREE CONDITIONS

Sample No.	Activity (Units/10^7 cells)	
	Lysates	Supernatants
1	0.7	8.2
2	0	9.8
3	0	0
4	0.2	8.8
5	0.6	0
6	0.9	6.4
7	0.5	0
8	0.2	2.1

TABLE 22

MEAN VALUES OF PLASMINOGEN ACTIVATOR ACTIVITY IN NORMAL AND TUMOUR CELLS LYSATES AND SUPERNATANTS DETERMINED BY FIBRIN PLATE ANAYLSIS

	Weeks in culture	Days in serum-free conditions	Activity (units/10^7cells)	
			Lysates	Supernatants
Normal Oral keratinocytes	2 weeks	1d	0.6	5.1
		2d	0.3	28.2
	4 weeks	1d	0.8	1.7
		2d	1.0	244.6
		4d	0.3	132.7
Normal Oral fibroblasts	2 weeks	2d	0.2	0
Tumour Oral keratinocytes	4 weeks	2d	0.4	4.4
Tumour fibroblasts	2 weeks	2d	0	0

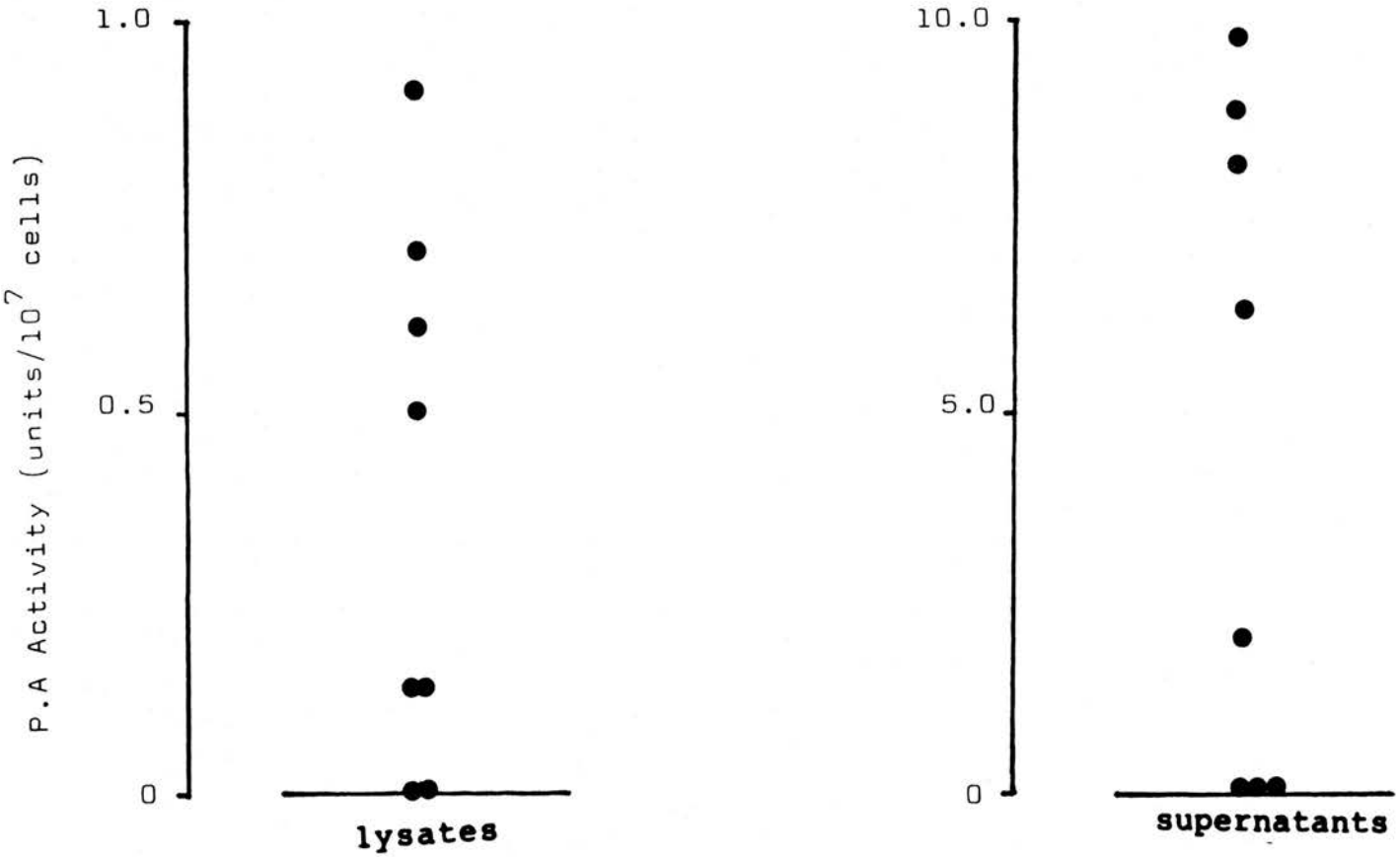


Fig.81 Plasminogen activator activity in cell lysates and culture supernatants from oral squamous cell carcinomas cultured for four weeks in vitro.

indicate that cell associated plasminogen activator activity from tumour cell lysates was low and was within the range of values found in cultured gingival epithelium (mean value $0.4U/10^7$ cells).

Plasminogen activator activity in cell free culture supernatants was absent in three of the eight cultures examined and overall activity remained low (mean value $4.4U/10^7$ cells). This activity was significantly lower ($p=0.05$ Mann-Whitney U Test) than that found in cultures of normal oral keratinocytes grown for four weeks *in vitro* (Table 22, Fig. 82).

3.3.5 Fibrin Plate Analyses of Normal and Malignant Oral fibroblast culture fluids

Cell associated plasminogen activator activity was detected in 2 of the five confluent cultures (passage 2) of fibroblasts from non-neoplastic gingival mucosa (Table 23). Activator activity in these cultures was less than $1U/10^7$ cells. No plasminogen activator activity was detected in any of the culture supernatants after two days in serum free medium.

Tumour cell lysates and culture supernatants from confluent cultures of fibroblasts (passage 2) contained no detectable plasminogen activator activity (Table 23).

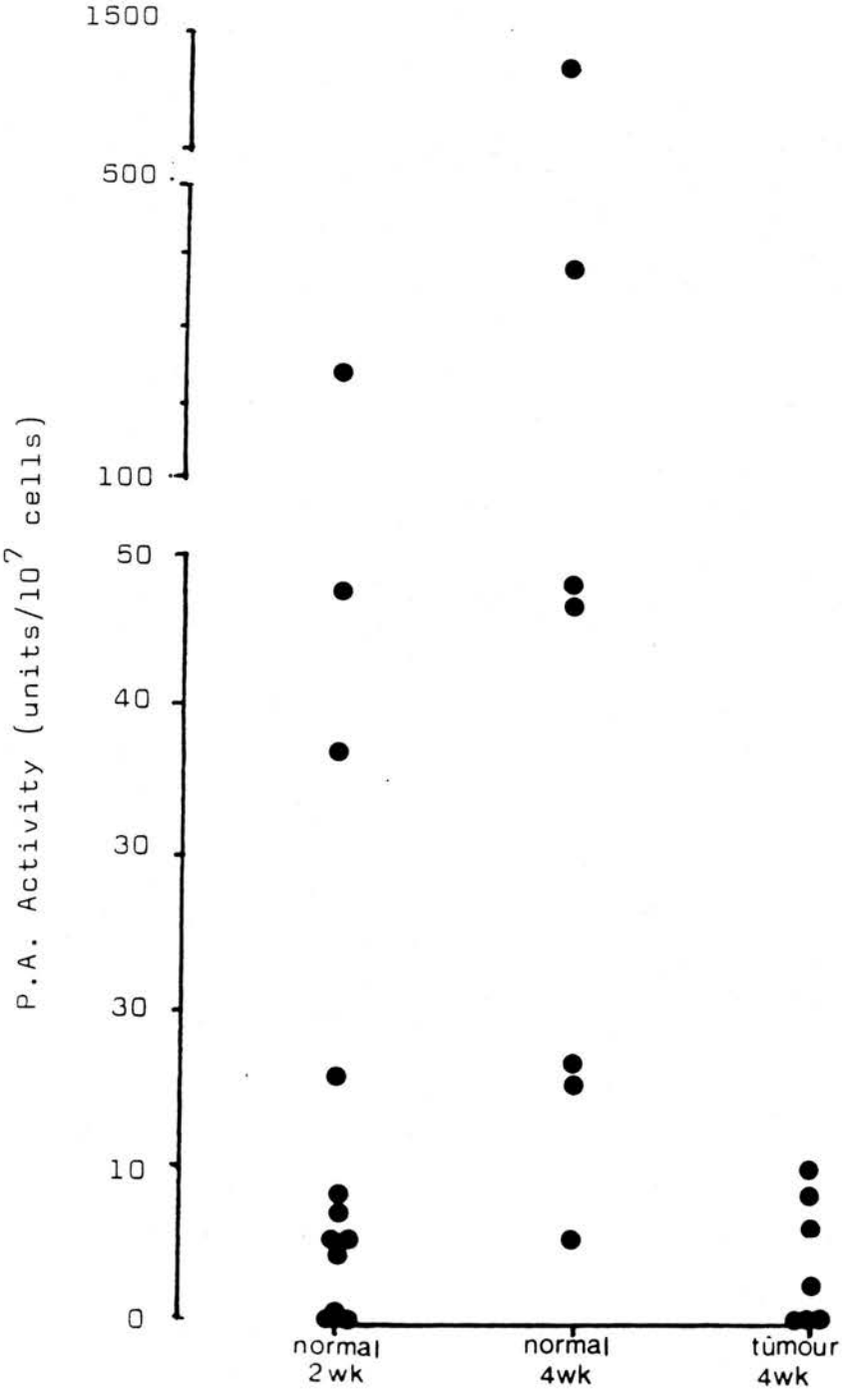


Fig.82 **Plasminogen activator activity in culture supernatants from normal oral keratinocytes cultured for two and four weeks in vitro and from oral squamous cell carcinomas cultured for four weeks in vitro.**

TABLE 23

FIBRIN PLATE ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL AND TUMOUR FIBROBLASTS FROM ORAL MUCOSA AFTER 4 WEEKS IN VITRO.

SAMPLES WERE COLLECTED AFTER 2 DAYS IN SERUM FREE CONDITIONS

	Sample N°	Activity (Units/10^7 cells)	
		Lysates	Supernatants
Normal Oral Mucosa Fibroblasts	1	0	0
	2	0.8	0
	3	0	0
	4	0.1	0
	5	0	0
Tumour Fibroblasts	1	0	0
	2	0	0
	3	0	0
	4	0	0

3.3.6 Analysis of Culture Lysates and Supernatants from Normal Gingival Keratinocytes by SDS-PAGE

Cell lysates from six cultures of normal gingival keratinocytes grown for two or four weeks *in vitro* were examined after SDS-PAGE. The activities of these samples measured by fibrin plate assay are shown in Table 24. In cell lysates, in which no activity was detected on fibrin plates, no activity was present in zymogram overlays of SDS-PAGE gels (Fig. 83). Absence of lysis was therefore not due to the presence of inhibitors in these lysates. In two lysate samples, which contained plasminogen activator activity ($<1U/10^7$ cells by fibrin plate assay) zones of lysis were apparent in the zymograms. These bands co-migrated with the uPA standard.

Of thirteen supernatants collected from cultures grown for two and four weeks *in vitro*, and which contained varying amounts of activity on fibrin plates (Table 24) all showed bands of lysis in zymograms which co-migrated with the uPA standard (Fig. 84). The observed bands of lysis could be inhibited by the incorporation of antibodies to uPA into the zymogram overlay gels. Four of the thirteen cultures also showed bands of lysis as a result of high molecular weight PA complexes ($M_r \simeq 200,000$) (Fig. 84). These areas of lysis were also inhibited by the inclusion of antibodies to uPA but not tPA in the zymogram overlay gels. The presence of high molecular weight bands did not appear to correlate with the culture age, the time after which the supernatants were

TABLE 24

ZYMOGRAPHIC ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY IN CULTURES OF NORMAL GINGIVAL KERATINOCYTES

Age of Culture (weeks)	Days in Serum-free Medium	Sample No.	Fibrin Plate Activity (a)	Activity present in Detector Gels (c)	
				LMW-uPA	HMW-uPA
2	1	(b) L1	0	-	-
2	2	L2	0	-	-
2	2	L3	0	-	-
2	2	L4	0	-	-
4	2	L5	1	+	-
4	2	L6	1	+	-
2	1	(b) S1	1	+	-
2	1	S2	1	+	-
2	1	S3	1	+	-
2	1	S4	1	+	+
2	2	S5	2	+	+
2	2	S6	3	+	-
2	2	S7	3	+	+
2	2	S8	3	+	-
4	1	S9	1	+	-
4	2	S10	4	+	-
4	2	S11	4	+	+
4	2	S12	4	+	-
4	2	S13	5	+	-

(a) Activity on Fibrin Plates
0 - No activity
1 - 0-1 $U/10^7$ cells
2 - 2-10 $U/10^7$ cells
3 - 11-20 $U/10^7$ cells
4 - 21-100 $U/10^7$ cells
5 - > 100 $U/10^7$ cells

(b) L = Lysates
(b) S = Supernatant

(c) - LMW Low Molecular Weight
- HMW High Molecular Weight

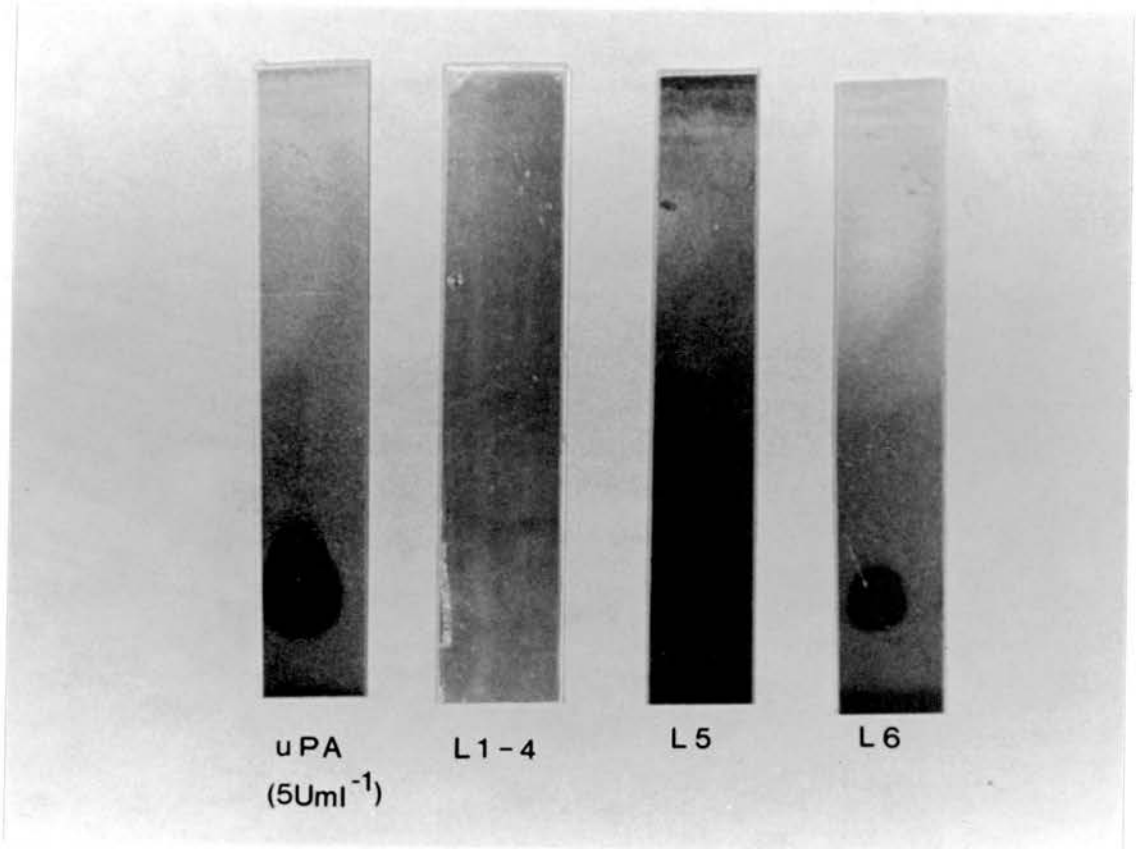


FIG. 83

Plasminogen activators in lysates from cultured gingival keratinocytes after SDS-PAGE.

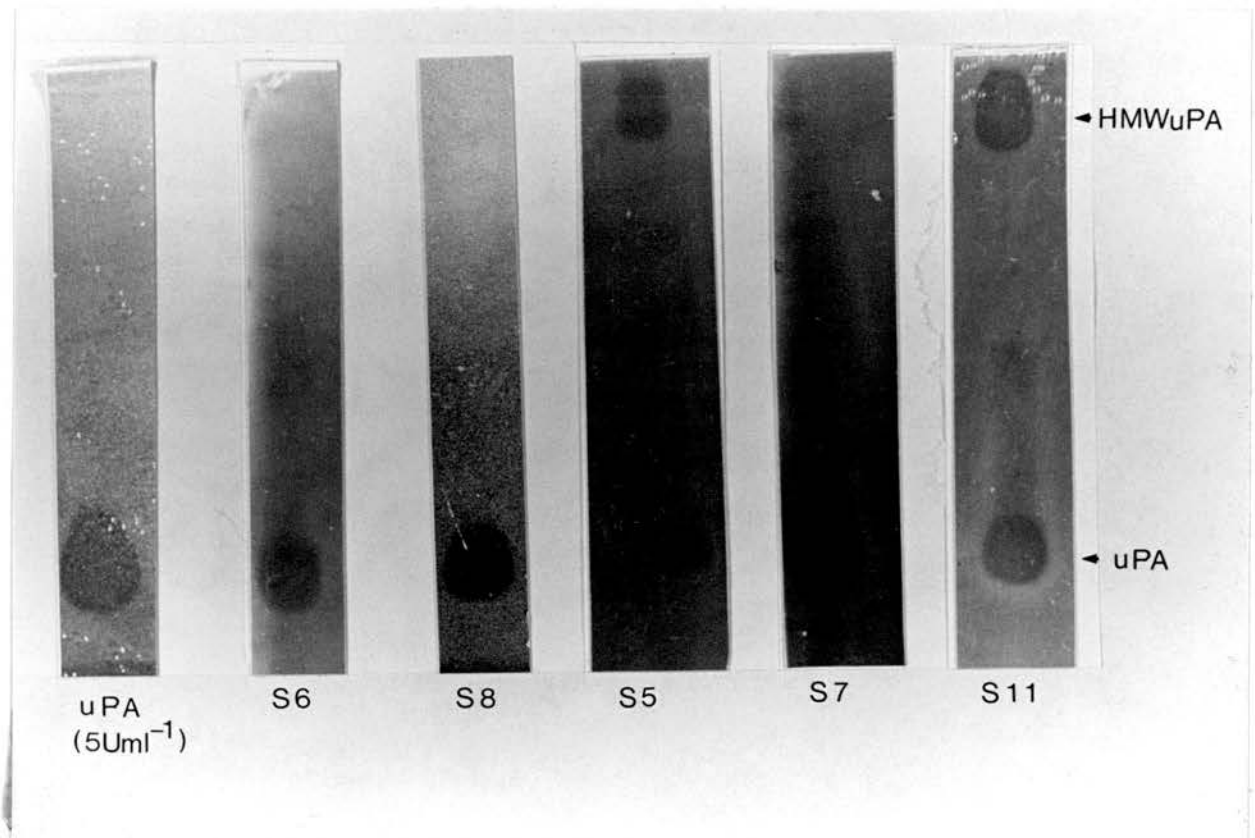


FIG. 84

Plasminogen activators in supernatants from cultured gingival keratinocytes after SDS-PAGE.

collected or the activity of the samples on fibrin plates (Table 24).

Of twelve samples which were assayed using plasminogen free zymogram overlay gels, none showed lysis, indicating that fibrinolytic activity was the result of plasminogen activator.

3.3.7 Analysis of Culture Supernatants from Tumour Keratinocytes by SDS-PAGE

Of the eight supernatants from keratinocytes derived from oral squamous cell carcinomas, five showed low fibrinolytic activity on fibrin plates (<10U/10^7 cells) and three showed no fibrinolytic activity (Table 21 + 25). After SDS-PAGE analysis of these supernatants using a 10 ul sample loading, very large zones of lysis were apparent in zymograms from two of the supernatants (T6 and T8) (Fig. 85) and these had to be diluted 1/10 (Fig. 86). When compared with supernatants of gingival keratinocytes, showing similar activities on fibrin plates, the areas of lysis on zymograms of the culture supernatants were much higher than those produced by gingival keratinocytes on zymograms. This suggests that plasminogen activator inhibitors (PAI) are present in the oral squamous cell carcinoma culture supernatants.

When the culture supernatants were diluted 1/5 or 1/10 before analysis with SDS-PAGE two of the supernatants T5 and T7 which had shown no activity on fibrin plates showed lysis

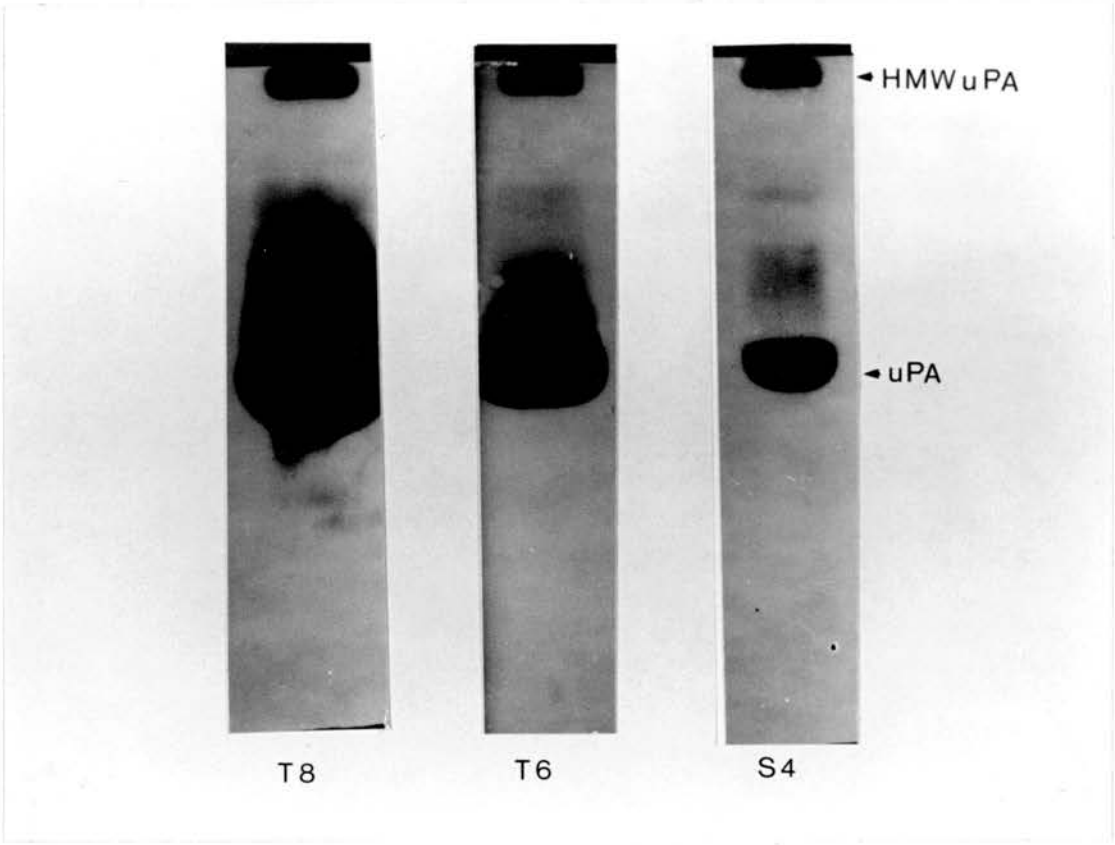


FIG. 85

Plasminogen activator activity in undiluted culture supernatants from oral squamous cell carcinomas (T6 and T8) after SDS-PAGE.

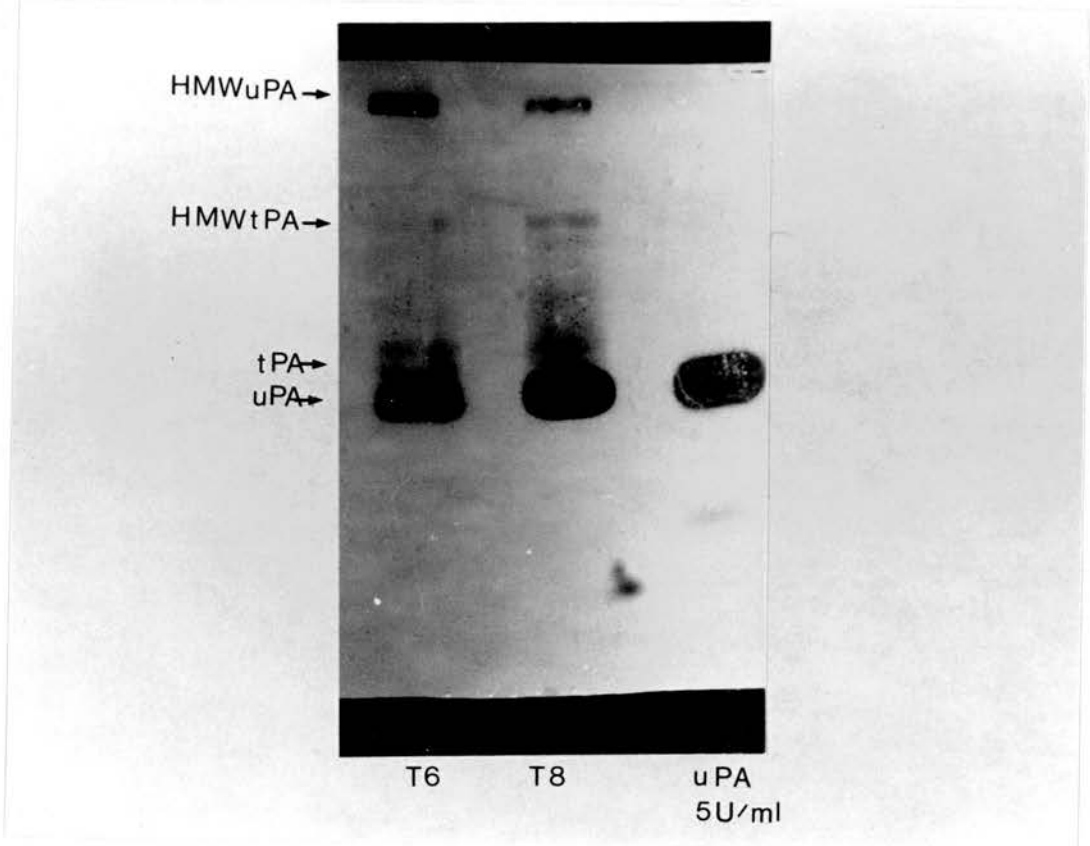


FIG. 86

Plasminogen activator activity in diluted culture supernatants from oral squamous cell carcinomas (T6 and T8)

in zymograms (Fig. 87). Only one tumour cell supernatant (T3) showed no activity on either fibrin plates or in zymogram detector gels. In all tumour supernatants (except T3) zones of lysis M_r≃55,000 were apparent and co-migrated with the uPA standards. These supernatants also showed bands of lysis as a result of high molecular weight PA-complexes (M_r≃200,000). These complexes could be PA-PAI complexes which show fibrinolytic activity only after SDS-PAGE manipulations. In one of the supernatants (T8) another band of lysis (M_r≃110,000) was also apparent in the zymogram gel (Fig. 88).

Further analysis of the tumour supernatants was carried out by incorporation of antiserum to uPA and tPA into the zymogram detector gels. The majority of fibrinolytic activity was quenched by incorporation of antiserum to uPA into gels (Fig. 88). Anti-uPA antiserum inhibited zones of lysis M_r≃55,000 and lysis due to the high molecular weight complex (M_r≃200,000) (Fig. 88).

Not all lysis was inhibited by antiserum to uPA. In all the supernatants (except T3) small bands of lysis were observed due to tPA activity. These bands were inhibited by the incorporation of antiserum to tPA (Fig. 88). In addition one tumour supernatant contained a high molecular weight complex M_r≃110,000. The fibrinolytic activity of this complex was quenched by the addition of anti-tPA antiserum into the zymogram. On the basis of its electrophoretic mobility and immunological identification it was presumed to be PAI-1 (Sprengers and Kluft, 1987).

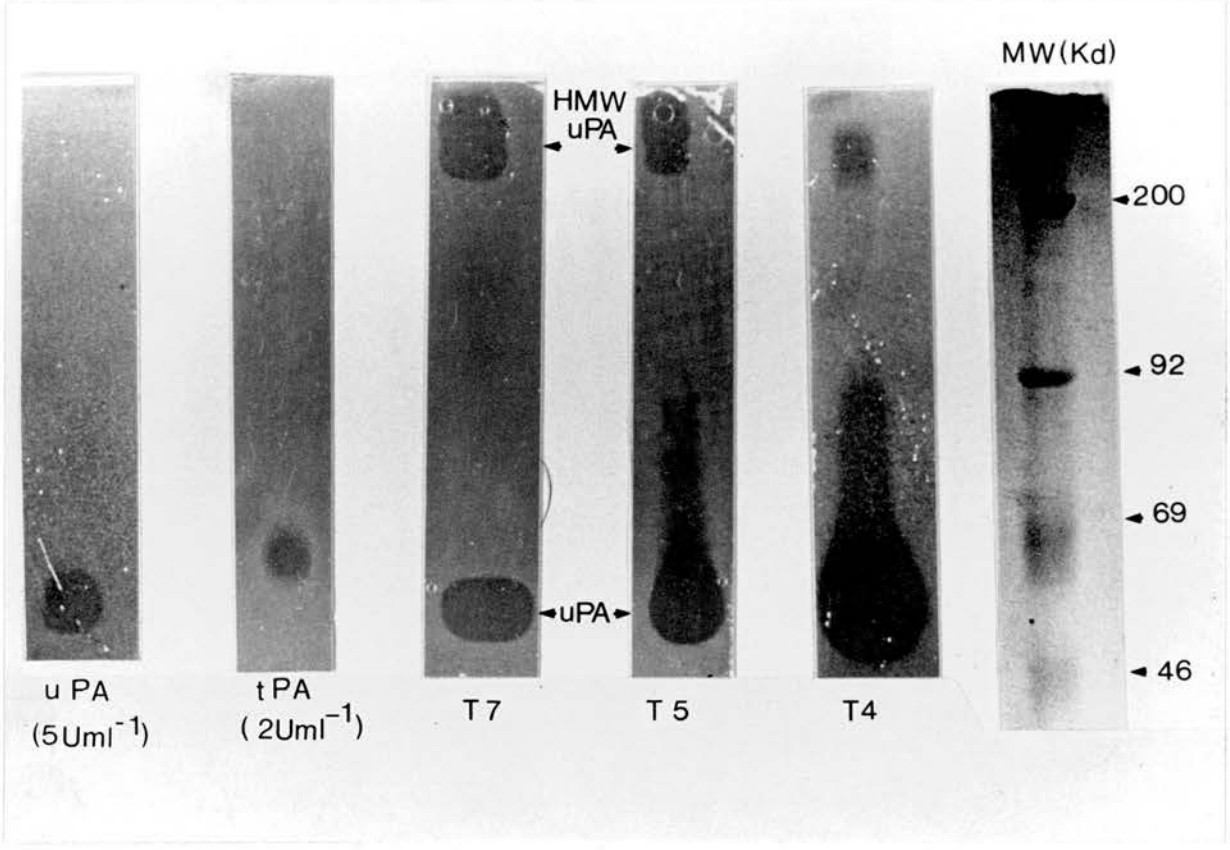


FIG. 87

Plasminogen activators in diluted culture supernatants from oral squamous cell carcinomas (T4, T5 and T7) after SDS-PAGE.

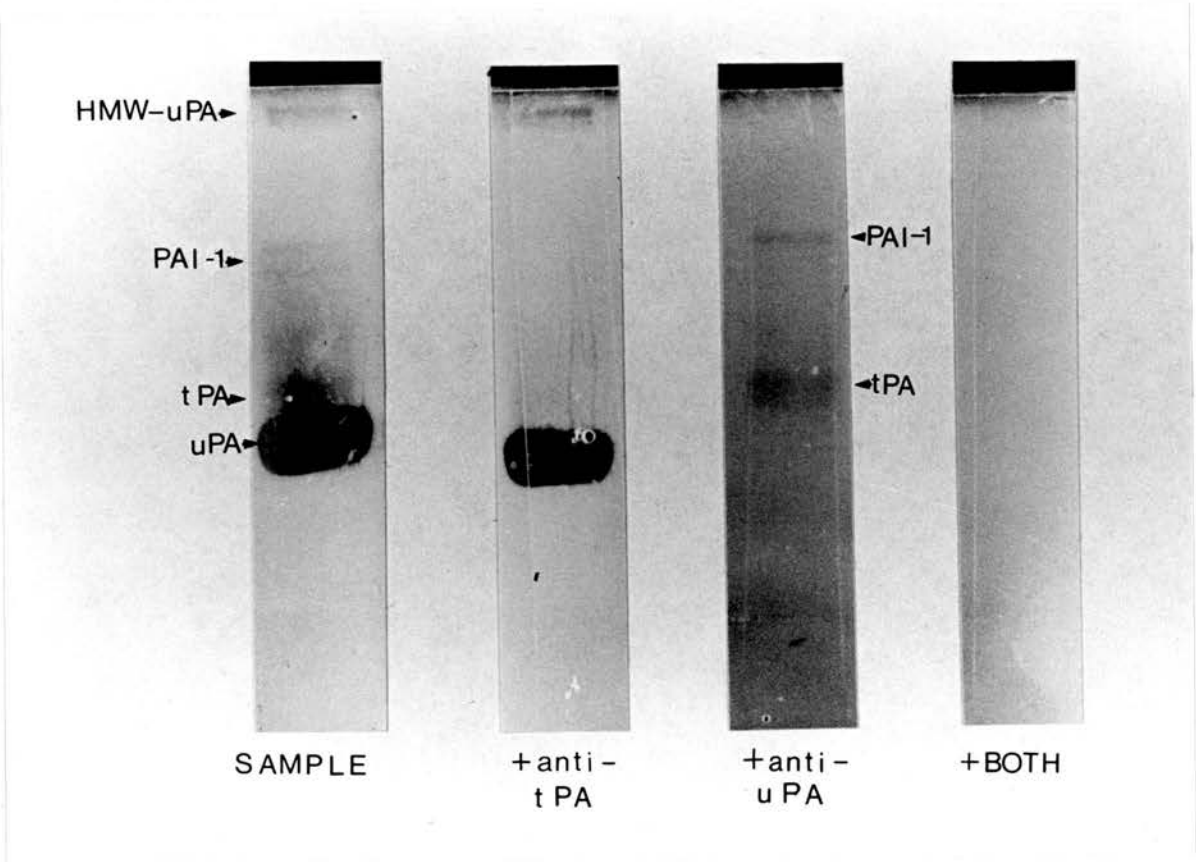


FIG. 88

Identification of plasminogen activators in tumour culture supernatant (T8) after incorporation of antiserum to uPA and tPA into zymogram overlay gels.

TABLE 25

ZYMOGRAPHIC ANALYSIS OF PLASMINOGEN ACTIVATOR ACTIVITY IN TUMOUR CULTURE SUPERNATANTS

Sample No.	Fibrin Plate Activity ($U/10^7$ cells)	Activity Present on Detector Gels		
		LMW-uPA	HMW-uPA	tPA
T1	8.2	+	+	+
T2	9.8	+	+	+
T3	0	-	-	-
T4	8.8	+	+	+
T5	0	+	+	+
T6	6.4	+	+	+
T7	0	+	+	+
T8	2.1	+	+	+

3.3.8 Analysis of Culture Lysates and Supernatants from Fibroblast Cultures from Gingival Mucosa and Oral Squamous Cell Carcinomas by SDS-PAGE

Two culture lysates and supernatants from fibroblasts from normal gingival mucosa, which contained no activity on fibrin plates, and oral squamous cell carcinomas were assayed after SDS-PAGE. No lysis due to uPA, tPA or high molecular weight PA complexes was detected in these samples.

Furthermore, in zymogram detector gels, in which plasminogen activator had been added to lyse the fibrin during the incubation period, no opaque zones due to the presence of free PAI could be detected in these samples.

3.4 DISCUSSION

In fibrinolytic autographs of gingival epithelium uPA and tPA were immunologically identified in crevice epithelium. Larger zones of lysis, due to the action of tPA were apparent over crevice epithelium but it is now well established that the activity of tPA is greatly enhanced by fibrin (Matsuo et al 1981; Hoylartes et al 1982), a property not shared by uPA. Thus smaller areas of lysis in crevice epithelium, as a result of uPA, may not reflect relative amounts of activator secreted by these cells.

Lysis was never found over keratinising gingival epithelium, a finding supported by others using human and rodent tissue (Southam and Moody, 1981; Southam et al 1981; Ljungner et al 1984). The absence of fibrinolytic activity may have been due to the absence of activators or it may arise from the presence of proenzymes to activators or plasminogen activator inhibitors (PAI) in these tissues. Proenzymes of uPA and tPA have recently been identified in human urine, plasma and cells in culture (Eaton et al 1983; Kasai et al 1985; Kielberg et al 1985). In common with other proenzymes, these are activated by limited proteolysis and this activation represents another step in the plasmin cascade at which control over proteolysis can be exerted. Fibrinogen preparations contain trace amounts of plasmin and therefore theoretically proenzyme, if present, should be detected in fibrinolytic autographs. Nevertheless, some commercial preparations of fibrinogen can contain trace amounts of inhibitors which can quench plasmin activity and

enrichment of the fibrinogen with plasminogen may be required (Gurewich et al 1984). Despite this many preparations of fibrinogen, in this and other work, have been used without detection of proenzyme activity in keratinising epithelium.

Immunofluorescence studies of gingival epithelium also indicated an absence of free plasminogen activator in keratinising epithelium. No fluorescence was observed anywhere in the epithelium after staining with antibodies to uPA and tPA. Positive staining was detected in vascular endothelium with both uPA and tPA. No staining for uPA or tPA was observed in crevice epithelium in these sections. It may be that plasminogen activators, shown to be present in fibrinolytic autographs, are leached from tissues during staining procedures. Furthermore, fibrinolytic activity is markedly enhanced by fibrin, thus low levels of tPA in sections may be sufficient to cause lysis in autographs, but may not be present in sufficient quantity to detect by immunofluorescence. Again, it is possible that plasminogen activators are present intracellularly as proenzyme. However, it has been noted that monoclonal antibodies to plasminogen activators can recognise determinants in the proenzyme form (Nielsen et al 1982). Thus proenzyme should also be detected if present. If plasminogen activators are present in keratinising gingival epithelium, they may be bound to inhibitors and these are unlikely to be detected by immunofluorescence or fibrinolytic autography. Inhibitors to both uPA and tPA have been found in cornified cells in skin

(Hibino et al 1984) and may also be present in keratinising layers of oral epithelium. Antibodies to PAI have recently been produced in another laboratory (Busso et al 1987) and these may help clarify a role for plasminogen activators in epithelium.

It is clear from this and other studies that plasminogen activators are present in certain areas of epithelium. Lysis due to plasminogen activators has been observed in epithelium in other areas of the mouth, most notably in lip, cheek and soft palate (Myhre-Jensen and Astrup 1971; Southam et al 1982) and lysis was observed to arise in the prickle cell layer. A number of roles for plasminogen activators in epithelium have been postulated. Green (1979), for example, found that nucleated squames collected from supernatants of cultured keratinocytes which were then further incubated, require the presence of plasminogen containing medium for nuclear digestion to occur. He suggested that normal differentiation and cellular dyshesion is a plasmin assisted process. This is difficult to reconcile with previous work which indicated that free plasminogen activator arises not in the cornified layers of epithelium but in the prickle cell layer (Southam et al 1981) and with the finding that cornified cells contain inhibitors to both uPA and tPA (Hibino et al 1984). It may be that the balance between activator and inhibitor production in vivo is sufficient to allow cellular dyshesion to occur, but that insufficient activator is generated in these tissues to be detected by fibrinolytic autography or immunostaining.

Further support for the role of activators in differentiation arises from *in vitro* work since levels of plasminogen activators have been shown to increase in cultured keratinocytes as they stratify and differentiate (Isseroff *et al* 1983; Birkedal-Hansen *et al* 1983; Morioka *et al* 1985).

Plasminogen activator has also been implicated in cellular migration. Morioka *et al* (1985) found abundant plasminogen activator in cells migrating at the edges of epithelial cultures. This has been confirmed in this study by fibrinolytic autographs of cultures of oral epithelium, in which zones of lysis were frequently detected at migrating edges of epithelial outgrowths. Morioka *et al* (1985) further showed that uPA accumulated in keratinocytes *in vitro* at the cut edges of wounds deliberately made in cultures. Other cell types including neurones migrating across culture surfaces also deposit plasminogen activator which Krystosek and Seeds (1986) considered a component of substrate adhesion material. It is possible that lysis arising in prickle cells in oral epithelium as a result of plasminogen activators is involved in migration of cells as they proceed from the basal to cornified layers.

Whatever the precise function of plasminogen activators in epithelium, their presence in oral epithelium (Southam *et al* 1981), glomerular and kidney tubule epithelium, ductus deferens and prostate epithelium (Camiolo *et al* 1984) and cultures or epithelium from numerous tissues (Dano *et al* 1985) would suggest that they are normal

components of epithelia and have a key role in the biology of normal epithelia.

In the present study no lysis was observed over tumour cells in fibrinolytic autographs of frozen sections of biopsies of oral squamous cell carcinomas. Lysis was mainly confined to vessel endothelium. This is consistent with the observation of Ljunger et al (1984) who also found no fibrinolysis over epithelial cells in autographs of oral squamous cell carcinomas. In other similar studies, fibrinolytic activity in autographs of endometrial adenocarcinomas, benign and malignant ovarian tumours and human colon tumours, lysis was also confined to blood vessels (Weiss and Beller, 1969; Swanberg et al 1975; Newstead et al 1976).

Immunofluorescence staining for uPA and tPA, performed on tissue sections from the same patient biopsies, showed very weak staining for uPA and tPA. Staining was infrequent and usually in isolated cells. Possibly low levels of antigen in tissues or complexing of plasminogen activators with PAI in tissues, combined with the monospecificity of the antibodies contributed to such poor staining in these sections. Despite the weakness of staining it was consistently present particularly after staining with anti-uPA antibodies, and was absent if staining was carried out with non-immune serum. Brighter fluorescence was observed in vascular endothelium after staining with both tPA and uPA and in connective tissue after staining with uPA. uPA has been detected in connective tissues by others (Larsson et al 1984)

and plasminogen activator production by tumour cells may not be a pre-requisite for invasion into surrounding tissues, if uPA is also present in connective tissue elements.

Few immunocytochemical studies using antiserum to uPA and tPA have been carried out. Of these, most have indicated that uPA is exclusively produced by tumour cells (Markus et al 1984; Khoga et al 1985) and these observations are consistent with many in vitro studies. Burtin et al (1985) demonstrated the presence of both uPA and tPA in tumour cells in human colon carcinomas. They found, using monoclonal antibodies to uPA and tPA that staining in tissues was weak and intermittent, but where staining did occur it was most intense in small isolated foci of tumour cells in the invasion zone and the staining was predominantly for uPA. No improvement in staining was obtained in these tissues when immunoperoxidase staining techniques were used.

In the studies of Markus et al (1984) and Khoga et al 1985) the authors achieved a more consistent staining of colon carcinomas using an immunoperoxidase staining technique. Abrupt changes in staining were seen in the transitional areas with staining intensity for uPA faithfully reflecting morphological transion to carcinoma. No tPA was isolated in tumour cells in these studies. The differences in patterns of staining in similar tumours may result from different antisera being used by different groups.

The presence of plasminogen activators in tumour sections does not necessarily indicate a role for such

enzymes in tumour invasion. However, their presence and evidence from many other studies that increased plasminogen activator synthesis is correlated with malignant transformation of cells makes this a reasonable hypothesis. Examples of this correlation are too numerous to cite (reviewed by Dano et al 1985), however, this relationship is not absolute (Chibber et al 1975; Wolfe and Goldberg 1976; Whur et al 1980; Hinuma et al 1985). In some tissues, particularly epithelium, both normal and malignant cells appear to contain high levels of plasminogen activator (Mott et al 1974; Wilson and Dowdle 1978; Wilson et al 1980). Wilson and Dowdle (1978) found in a series of cultures derived from normal, reactive and neoplastic tissues, that there was no consistent relationship between plasminogen activator production and neoplasia. However, in the light of recent discoveries of proenzymes and plasminogen activator inhibitors, much of this type of data needs to be re-evaluated.

Unlike fibrinolytic autographs of cultured gingival epithelium, all of which showed extensive lysis particularly at the migrating edges of epithelial outgrowths, lysis in oral squamous cell carcinomas was observed only in a small group of cells in an epithelial outgrowth from one patient biopsy. Again, this apparent lack of plasminogen activator activity was unexpected, particularly in view of the numbers of reports emphasising the increase in plasminogen activator synthesis in cell cultures of neoplastic origin and in transformed cell lines (Dano et al 1985). However, some cell

cultures of neoplastic origin have been found to lack detectable plasminogen activator activity especially when analysed soon after the establishment of the culture (Wilson and Dowdle 1978; Gronow and Blenheim, 1983). In the present study, it cannot be evaluated from fibrinolytic autographs alone whether low fibrinolytic activity is due to production of small amounts of plasminogen activators or the concomitant release of plasminogen activators and inhibitors.

Epithelium grown in vitro from gingival mucosa and oral squamous cell carcinomas allowed comparisons of the levels of plasminogen activators in near pure populations of non-neoplastic and tumour epithelium.

The medium used to culture keratinocytes contained epidermal growth factor, which is also present in small quantities in serum. Epidermal growth factor is known to induce plasminogen activator synthesis (Lee and Weinstein, 1987) but PA synthesis is rapidly down regulated after the removal of EGF (Gross et al 1983b). Serum has also been shown to contain other modulators of plasminogen activator activity in addition to growth factors, hormones and plasma protease inhibitors (Levin and Luskotoff, 1980). These authors found, using bovine aortic endothelial cells, that plasminogen activator activity increased with cell density, but decreased by 70% - 80% after the addition of fresh serum during routine feeding. No such inhibition was observed if cultures were simply washed and the same medium returned to the cells, or by the addition of other proteins. The authors

postulated that plasminogen activator production by bovine aortic endothelial cells *in vitro* was subject to negative control by suppressors within serum. By dialysing or acid treating serum, it was found that neither steroid hormones nor serum inhibitors were involved in the suppression of plasminogen activator activity and the identity of the molecule(s) remains unclear. However, suppressors must in some way be inactivated or degraded during cell growth, the cells thus being released from the suppressed state. In view of these findings, plasminogen activator production by oral epithelial cells was determined in basal medium containing only 0.5% bovine serum albumin.

Proteolytic activity in culture fluids was initially assessed by the fibrin plate method. Plasminogen activator activity was plasminogen dependent indicating that lysis was due to plasminogen activators and not other non-specific proteases.

Not all cell lysates expressed plasminogen activator activity on fibrin plates and in lysates which did contain activity, little difference was observed after two or four weeks growth *in vitro*. Levels of cell associated activity were always low.

The majority of plasminogen activator detected in cultures was secreted into the culture medium. Levels of plasminogen activator in cultures of gingival keratinocytes grown for two weeks *in vitro* increased four fold in supernatants collected after two days, compared to those collected after one day in serum free medium. It may be that

there is a lag phase for the synthesis of plasminogen activators. Indeed, inclusion of the protein synthesis inhibitor, cycloheximide, into serum free supernatants completely ablated production of plasminogen activator in cultures. Plasminogen activators must therefore be synthesised *de novo*.

Gingival keratinocytes after four weeks *in vitro*, secreted far higher levels of plasminogen activators into supernatants over a two day period, than two week old cultures. This is in agreement with the findings of others (Birkedal-Hansen *et al* 1983; Morioka *et al* 1984) and again suggests the involvement of plasminogen activator in some aspect of terminal differentiation in oral epithelial cultures. Despite a ten fold difference in the levels of plasminogen activators observed in four week old cultures compared to two week old cultures, this increase was not statistically significant and probably reflects the large inter-patient variation. Wilson and Dowdle (1978) also noted large inter-sample variation in cultures of epithelium from a number of tissues. This was particularly pronounced in kidney epithelial cultures in which activities ranged from 190U - 3,700U/10^7 cells/24h. Levels of activity found in gingival epithelial cultures ranged from 23U - 1427U/10^7 cells and were comparable to those found in Wilson and Dowdle's study. Wilson and Dowdle attributed their findings to experimental variables; however, in the absence of sufficient patient data it is difficult in this study to

speculate whether these differences reflect innate *in vivo* differences, correlated with patient variables such as donor age and health or whether they result from experimental variables. Certainly, despite all attempts to keep protocols uniform, Wilson and Dowdle found that not only did plasminogen activator levels vary substantially in primary cultures, but these also varied markedly in the same cultures after routine passage. No attempts were made in their study to evaluate levels of inhibitors in these cultures.

Cell associated plasminogen activator activity in four week old cultures of tumour derived keratinocytes did not differ greatly from levels of activity found in normal keratinocyte cultures. Plasminogen activator activity in tumour supernatants was, however, significantly lower (Mann-Whitney U Test $p=0.05$) than that measured in normal culture supernatants by the fibrin plate method. These findings contradict the majority of data concerning plasminogen activator production by cells both *in vivo* and *in vitro*, in which a strong positive correlation has been observed between malignant transformation and increased synthesis of plasminogen activators (Ossowski *et al* 1973; Unkeless *et al* 1974; Soszka and Olzsewski 1986). These comparisons have often been made using normal and virally transformed fibroblast cell lines. Fibroblasts *in vitro* generally produce little or no detectable enzyme whereas epithelial cultures produce varying amounts of enzyme (Wilson and Dowdle 1978). Differences in plasminogen activator activity between normal and neoplastic epithelium may therefore be less

pronounced.

In addition, fibrin potentiates tPA activity and variations in plasminogen activator activity produced in assays may result from the use of a fibrin based method of detection. In the present work the use of a non-fibrin based assay, the S2251 chromogenic substrate assay, was investigated. The cleavage of bonds in the substrate by plasminogen activator generated plasmin, releasing p-nitroanaline, which was measured at 405 nm. However, phenol red pH indicator, which is added to most commercially available media, interfered with absorbance readings at this wave length. In the presence of phenol red indicator, over the pH range 7.0 - 7.4 no marked differences in absorbance readings were observed. However, at pH7.4 and above, a marked interference in absorbance readings was demonstrated in phenol red containing medium. The adjustment of pH in small samples of culture supernatant in this work was not feasible so S2251 substrate was not used to assay the activity in the culture specimens.

Neither the fibrin plate nor the S2251 assay distinguish between the types of plasminogen activators present in the samples being tested. Furthermore, activity in the samples may be masked by the presence of PA inhibitors. The separation of activators from inhibitors and further immunological identification of activators was therefore carried out by SDS-PAGE. Samples of cell lysates and supernatants from normal gingival keratinocytes

displaying a range of activities on fibrin plates, were analysed after SDS-PAGE. Of the six cell lysates examined in this way only those samples which expressed fibrin plate activity showed zones of lysis on zymogram detector gels. These bands co-migrated with the urokinase standard. No other lytic bands were detected in the zymograms of epithelial cell lysates even after prolonged incubation (4 days). The absence of fibrin plate activity therefore appeared to be due to the absence of cell associated plasminogen activator activity in these samples. No definitive assay of pro-activator activity in these samples was undertaken but it would seem unlikely that proenzyme would remain unactivated by plasmin contaminants in plasminogen enriched detector gels. The plasminogen added to overlay gels was known to contain approximately 0.01 unit of plasmin per unit of plasminogen, in addition to the plasminogen already present in the fibrinogen preparation.

Thirteen supernatants from cultures of gingival keratinocytes were also analysed by SDS-PAGE. These supernatants had a wide range of fibrin plate activities (<1U - >1000U/10^7 cells). All cell culture supernatants displayed bands of lysis which co-migrated with the urokinase standard (M_r=55,000). This activator was positively identified in these samples by the inclusion of antibodies to urokinase into the detector gels. No activity due to tPA was apparent.

Faint bands of lysis, at a position in the gel indicating a much lower electrophoretic mobility, were present in four of the thirteen samples. These bands could

all be inhibited by anti-urokinase antibody. The presence of these bands was not obviously correlated with the age of the culture, the time at which the samples were harvested or the fibrinolytic activity expressed on fibrin plates.

Several other groups have shown that primary cultures of normal human foreskin keratinocytes produce mainly uPA (Wilson et al 1980; Hashimoto et al 1985; Morioka et al 1985). In addition Wilson's group showed that uPA was the major activator in cultures of bladder, thyroid, skin and oesophageal epithelium and in mixed cultures of glial and neuronal elements. Bands of lysis relating to activators with lower electrophoretic mobilities were also observed in a minority of their cultures. The appearance of these bands has been attributed to dimerisation or aggregation of the lower molecular weight forms of activators. However, Cieplak and Hart (1986) have shown that these bands are stable in SDS and resistant to reduction or thermal degradation. These complexes, therefore, would not appear to represent aggregates or disulphide linked dimers or oligomers.

As a result of mixing experiments using radio-iodinated urokinase, Cieplak and Hart (1986) found, in human urine, that the presence of high molecular weight forms of PA were the result of complexes between low molecular weight forms of PA and a binding component. Electrophoretic analysis of ^{125}I-labelled high molecular weight uPA complexes (HMWuPA) suggested that the binding component had an approximate M_r of 40,000. An inhibitor role was suggested for the binding

component, as serine protease inhibitors were unable to inhibit the formation of these complexes.

A number of inhibitors have been suggested to form these complexes. Protease nexins released by fibroblasts have an M_r≃40,000 and bind covalently to uPA and thrombin (Baker et al 1980). Hoal et al (1983) also identified an M_r ≃40,000 - 50,000 molecule produced by fibroblasts, which forms SDS stable complexes with tPA, and is therefore functionally distinct from protease nexin. More recently, specific fast acting inhibitors which form stable complexes with uPA and tPA have been identified in human plasma (Sprengers and Kluft 1987).

These PA-PAI complexes have no inherent fibrinolytic activity. However, after manipulations performed during SDS-PAGE the complexes exhibit activity on zymogram overlays. It is unclear whether this is due to some dissociation of the complexes or intrinsic activity after SDS-PAGE. Such complexes often require more prolonged incubation for their presence to be observed as they express much lower activity than an equivalent amount of free activator.

Plasminogen activator inhibitors are produced by a number of cell lines including epithelial cells, endothelial cells, human hepatocytes, hepatoma lines, granulosa cells and melanoma cell lines (Wilson et al 1980; Van Mourik et al 1984; Sprengers et al 1985; Ny et al 1985; Schleef et al 1985). It remained a possibility that some of the inhibitor present in cultures was derived from contaminating fibroblasts in epithelial cultures. Fibroblast contamination

estimated in epithelial cultures used for plasminogen activator assays was low < 3% of the area of the culture.

In the present study, very low amounts of cell associated plasminogen activator ($\bar{x}$ = < 0.2 U/10^7 cells) were detected in lysates from only two pure fibroblast cultures of gingival mucosa by the fibrin plate assay. Also no PA activity was detected in any of the supernatants. The contribution of plasminogen activators by fibroblasts *in vitro* must, therefore, be very low. In those samples expressing no cell associated fibrinolytic activity, no plasminogen activator or PAI complexes were detected by SDS-PAGE in either supernatants or lysates. However, it is possible that uncomplexed inhibitor might be produced by fibroblasts in the cultures. In order to detect free inhibitor in fibroblast culture fluids, zymogram overlays containing urokinase were applied to SDS-PAGE gels. No free inhibitor was found in samples from pure cultures of fibroblasts. The contribution of plasminogen activators or PA-inhibitors produced by fibroblasts to the overall activity displayed by epithelial cultures must therefore be negligible.

Plasminogen activators were identified in all tumour supernatants, with the exception of one sample T3 which will be excluded from further discussion. Plasminogen activators were so abundant in tumour samples after SDS-PAGE separation of activators from inhibitors, that all samples had to be diluted five to ten fold before assaying. All the tumour

supernatants contained low molecular weight uPA which co-migrated with the urokinase standard (M_r≃55,000). These bands were positively identified in all tumour supernatants by the inclusion of antibodies to uPA into the zymogram detector gels. All of the tumour supernatants analysed in this way also contained much smaller amounts of free tPA resulting in faint bands of lysis with an M_r≃70,000. These bands were only narrowly separated from uPA bands and were positively identified by the inclusion of antibodies to either uPA or tPA into the detector gels. Despite the strong association between the production of uPA in tumours (Markus et al 1984; Camiolo and Greco 1986) and in tumour and transformed cell cultures (Dano et al 1980; Wilson et al 1980; Mira-y-Lopez et al 1983; Ossowski et al 1983), a number of reports indicate that uPA may not be uniquely produced by tumour cells in vitro (Wilson et al 1980; Schleuning and Reich 1983; O'Grady et al 1985). Wilson et al (1980) characterised plasminogen activators in neoplastic cultures from a number of carcinomas and found that while the predominant activator was uPA, in a small number of supernatants tPA was also identified. Similarly O'Grady et al (1985) found that benign breast tumours and carcinomas both contained uPA and tPA, but uPA was significantly higher in the malignant group.

Tissue plasminogen activator was identified by immunofluorescence in the present study, in isolated cells in sections of oral squamous cell carcinomas, from which cell cultures were derived. It would therefore seem reasonable to

postulate that epithelial cells may be capable of synthesising tPA *in vitro*. There is a possibility that small amounts of tPA could be secreted by other cell contaminants (for example leucocytes), in epithelial cultures derived from oral squamous cell carcinomas. However, in view of the numbers of these cells present in cultures this seems extremely unlikely. Furthermore, similar cells formed contaminants of gingival keratinocyte cultures where no tPA was detected in supernatants.

Unlike culture supernatants from gingival keratinocytes, all tumour supernatants showed the presence of one or more bands with lower electrophoretic mobilities which caused lysis in detector gels. These PA-inhibitor bands (M_r ≈200,000) caused zones of lysis which were larger than those found in supernatants from gingival epithelium and were inhibited by anti-urokinase antibodies. In one tumour supernatant a narrow band with an approximate M_r of 110,000 was detected in zymograms. This was inhibited by antibodies to tPA and was presumed to be PAI-1.

The nature of the inhibitor complexed to uPA is uncertain and further work is required to establish the precise M_r of these complexes. Recently it has been reported by Booth *et al* (1987) that an inhibitor complex with an apparent M_r of 180,000 is present in blood samples from volunteers after exercise and this was shown to be due to a complex with C1 inhibitor; however, this complex was tPA related. Complexes with alpha-2 macroglobulin with a similar

M_r have also been detected using these techniques (Booth, pers. comm.).

The possibility that significant amounts of inhibitor could be synthesised in these cultures by tumour fibroblasts, which are sometimes present as a very minor contaminant (< 2 % cover), was excluded after assaying culture fluids from pure cultures of tumour fibroblasts. None of the tumour fibroblast lysates or supernatants contained plasminogen activator, PA-inhibitor complexes or free inhibitor after SDS-PAGE analysis. PA inhibitors in these cultures must therefore be synthesized by epithelial cells.

In Summary

Plasminogen activators, uPA and tPA, were detected in sections of normal oral epithelium by fibrinolytic autography, where they may function in cell migration or terminal differentiation of epithelium. Although the precise role of plasminogen activators in epithelium remains unclear, their presence in oral epithelium and epithelium from other anatomical sites suggests some normal function of plasminogen activators in this tissue.

Plasminogen activators could not be detected, in fibrinolytic autographs over sections of oral squamous cell carcinomas, in epithelial cells. However, very weak immunostaining for uPA and tPA, in occasional epithelial cells in these sections, was observed and it may be that concomitant secretion of inhibitors in tumour tissue masks

the presence of plasminogen activators in oral squamous cell carcinomas.

Based on the observations of others, the hypothesis that increased plasminogen activator activity is correlated with neoplasia, was investigated using an *in vitro* model in which levels of plasminogen activator in virtually pure populations of normal and malignant oral keratinocytes could be compared.

In fibrinolytic autographs of cultured epithelium, extensive lysis was observed in all cultures of normal gingival keratinocytes, while lysis was virtually absent in cultures of tumour epithelium. This observation was confirmed when plasminogen activators secreted by normal and malignant epithelium were assayed by the fibrin plate method. Plasminogen activator activity was significantly lower in tumour cultures than in gingival keratinocyte cultures after four weeks growth *in vitro*. Since all supernatants from gingival keratinocyte cultures contain uPA, and since uPA was the predominant activator in tumour cultures, the differences in enzyme activities were not as the result of a tPA bias introduced by using the fibrin plate method.

After SDS-PAGE analysis of the culture fluids it became apparent that, once plasminogen activators were separated from inhibitors, tumour supernatants contained abundant plasminogen activator activity which was masked by the presence of inhibitors in these cultures. Unlike normal gingival keratinocytes, which secreted uPA, tumour cultures secreted both activators but predominantly uPA. PA-inhibitor

complexes were also detected in all tumour supernatants but the identity of the precise inhibitors involved is still uncertain.

If PA secretion by tumour cells is involved in tumour invasion, then it seems reasonable to suggest that it must be present in sufficient quantities to facilitate the process of invasion. PA-inhibitor production by tumour tissue may therefore cast doubt on the role of plasminogen activators in the tumour invasion zone. However, it has been shown that various hormones eg. EGF, TGF-beta and calcitonin can stimulate PA synthesis (Lee and Weinstein, 1978; Dayer et al 1981; Sudol 1985; Laiho et al 1986). Since some tumour cells are capable of synthesizing these hormones the balance between PA and PAI synthesis may be altered in vivo. Furthermore, the finding that plasminogen readily binds to a number of cell types and to extracellular matrix, and that its cleavage to plasmin once bound on these surfaces protects plasmin from inhibitors (Silverstein et al 1985b; Knudsen et al 1986) suggests that the overall amount of inhibitor present in tissues may not limit the process of tumour invasion.

In this study the presence of plasminogen in sections of gingival mucosa and oral squamous cell carcinoma was investigated.

CHAPTER 4

PLASMINOGEN IN ORAL EPITHELIUM

4.1 INTRODUCTION

The process of tumour invasion is generally considered to involve the production and release of proteases capable of degrading basement membrane components (Liotta et al 1985), and plasmin generated by proteolytic cleavage of plasminogen by activators has been assigned a major role in the breakdown of these complex matrix components (Jones and DeClerk, 1980, Laug et al 1983; Mignatti et al 1986). The sites of synthesis and storage of plasminogen are therefore crucial to the question of tumour invasion.

The major site of synthesis of circulating plasminogen has still not been firmly established, although one study has identified the liver as the major organ of plasminogen synthesis in man (Raum et al 1980). In terms of neoplastic invasion localised synthesis or storage of plasminogen within or near the location of the tumour may be of greater importance. Few reports on the localisation of plasminogen to sites other than the liver have been reported and these include eosinophils (Barnhart and Riddle, 1963), granulocytes (Prokopwicz and Stormorken, 1968), epidermis (Isserof and Rifkin, 1983; Nakagawa et al 1984) and vascular and extracellular areas outside seminiferous tubules (Saksela, 1986). Such studies indicate that localised plasminogen synthesis can take place in vivo and these

reservoirs of plasminogen may be involved at sites of tumour invasion.

De novo synthesis of plasminogen need not be a prerequisite for fibrinolytic activity at these sites. Plasminogen has been shown to bind to the surface of a number of cell types and to extracellular matrix elaborated by cells in vitro (Bogenmann and Jones, 1983; Miles and Plow, 1985; Hajjar et al 1986; Knudsen et al 1986).

Hajjar et al (1986) found that glu-plasminogen binds to human umbilical vein endothelial cell (HUVEC) monolayers in vitro. Binding occurs in a concentration dependent manner and is saturable at physiological concentrations (2uM). Plasminogen binding to these cells was 70-80% inhibited by eACA which suggests that binding is mediated through lysine binding sites. In their system plasminogen was activated by tPA in a high affinity-enzyme interaction (Km=5.9nM), and plasminogen on the cell surface was activated with a 13 fold greater efficiency than fluid phase plasminogen. Similar results have been reported in the circulatory system (Bachmann and Kruithof, 1984). Fluid phase plasminogen activation is kinetically unfavourable and immobilisation of plasminogen on fibrin clots via lysine binding sites makes activation of plasminogen possible at circulating concentrations. Such a mechanism, both in the vascular system and within tissues, may serve to promote and localise plasminogen activator activity. Plasminogen binding was found, by Hajjar et al (1986), to be relatively surface

specific with lower levels of binding to human fibroblasts, poor binding to smooth muscle cells (SMC) and no binding to bovine endothelial cells, indicating that species specificity may be critical in this interaction. They also found that the binding of unrelated proteins, such as albumin, to HUVEC layers was negative although other kringle containing proteins with lysine binding capacities were positive. Miles and Plow (1985) have reported that plasminogen also specifically binds to both resting and thrombin stimulated platelets in vitro. Again, plasminogen bound to the surface of the platelets was activated much more readily by tPA, uPA and SK than fluid phase plasminogen. Plasminogen also bound to granulocytes and lymphocytes present in their in vitro system. Miles and Plow postulated the presence of a fibrinogen related plasminogen receptor based on the number of plasminogen binding sites. No such binding sites were detected on HUVEC monolayers as fibrinogen could not be immunologicaly identified on the cell surfaces. The plasminogen binding sites on HUVEC were unidentified (Hajjar et al 1986). It is worth noting that not all authors have demonstrated plasminogen binding to cells (Bauer et al 1984) although the use of sub-physiologic concentrations of plasminogen and the use of human plasminogen with porcine endothelial cells may explain this discrepancy.

Knudsen et al (1986) further reported that plasminogen was capable of binding not only to cells but also to extracellular matrix (ECM) synthesised in vitro by endothelial cells. Such cells secrete a complex array of

glycoproteins and glycoasminoglycans which contribute to the formation of basement membranes (Martinez-Herandez and Amenta, 1983) and these matrix constituents include fibronectin, laminin, collagen and thrombospondin. Knudsen's groups previously demonstrated, in a fibrin free system, that plasminogen binds with high affinity to thrombospondin (Silverstein et al 1985a) and to histidine rich glycoproteins (Silverstein et al 1985b) and that tPA specifically bound to the thrombospondin-plasminogen complex (Leung et al 1984). Using the more biologically relevant substrate ECM, they have reported that plasminogen binding was specific, saturable at physiological concentrations and was lysine binding site dependent. Activation of plasminogen in this system was equally efficient by tPA and two chain uPA and, as expected, surface bound plasminogen was a much better substrate than fluid phase plasminogen. Of particular importance to neoplastic invasion was the finding that plasmin generated on the ECM surface was protected from the fast acting inhibitor alpha-2-antiplasmin. This would result in the prolongation of proteolytic activity on the matrix since fluid phase plasmin is readily inactivated by a number of inhibitors (Wiman and Collen, 1977). Of interest, too, was the observation that N-terminal cleavage of Glu to Lys plasminogen occurred quickly in this system. Lys-plasminogen is a much better substrate for tPA and, even in the fluid phase, considerable activation can take place (Wiman and Collen, 1977). Efficient generation of plasmin on ECM may be

due to a much higher Lys/Glu ratio. Several investigations have shown that neoplastic cells are capable of degrading endothelial or SMC generated matrices and that such breakdown is usually plasminogen dependent (Jones and DeClerk, 1983; Laug et al 1983; Bogenmann and Jones, 1983) although this is not always the case (Kramer et al 1982).

Plasminogen has been detected by immunofluorescence staining in the basal layer of skin (Isseroff and Rifkin, 1983) but there are no reports of its location in oral mucosa. In the present study, the localisation of plasminogen in normal oral mucosa and oral squamous cell carcinomas was undertaken by immunoperoxidase and immunofluorescent staining techniques.

Chemical alteration of tissue during routine paraffin embedding procedures often reduces reactivity of tissues during subsequent immunoperoxidase staining. Fixation, particularly by cross-linking fixatives, is known to reduce the antigenicity of some tissues (Pearse, 1980; Mepham, 1982). Clearing agents such as xylene also have an extracting process on cell cytoplasm (Zeitoun and Lehy, 1970) and a deleterious effect on tissue immunoreactivity (Matthews, 1981). Despite these drawbacks many antigens frequently survive formaldehyde fixation and paraffin embedding (Burns et al 1974). The use of frozen (cryostat) sections avoids the problems of chemical alteration of tissues during paraffin embedding procedures. However, although immunofluorescence can be carried out on unfixed sections, in practice, fixation is often necessary to

maintain the integrity of the tissue during staining. Immunoperoxidase staining of paraffin embedded sections, however, has definite advantages over immunofluroescence staining using frozen sections: for example, permanent preparations are obtained which can be studied by conventional light microscopy, better morphological preservation is achieved than with cryostat sections and retrospective studies can be performed.

Immunoperoxidase staining was, therefore, used initially in the present study in attempts to determine the presence of plasminogen in sections of oral mucosa.

4.2 IMMUNOPEROXIDASE STAINING

4.2.1 Materials and Methods

Specimens of oral mucosa (12), salivary gland (4), breast (2), and kidney (1) were fixed overnight in either 10% neutral buffered formalin or Bouin's fixative and processed for paraffin embedding. Sections were cut at 5 um and placed on slides pre-cleaned in chromic acid and coated with 0.1% poly-L-lysine (MW 24,000; Sigma) and dried for at least 24h at 37°C. Sections were deparaffinised in two changes of xylene and processed through alcohol to 70% methanol.

Endogenous peroxidase activity was blocked by placing the slides in 3% H_2O_2 in methanol for 30 min and then washing in running tap water for 5 min. Non-specific binding of unwanted antibodies in the serum was inhibited by pre-incubation of the slides in a 20% solution of normal swine serum (Flow Labs) in Tris buffered saline (TBS) pH 7.6 for 15-30 min. This solution was drained off the slides and the primary antibody-antiserum to human plasminogen, raised in rabbits (Dakopatts), was applied to the sections at dilutions from 1:25 to 1:200 for 30 min at room temperature. The sections were then washed in two changes of TBS for 15 min. and the bridging antibody, swine anti-rabbit Ig (Dakopatts), was applied to the sections in a 1:30 dilution for 30 min. The sections were again washed in two changes of TBS for 15 min. and covered with rabbit antiperoxidase-peroxidase conjugated antibody (Dakopatts) at a 1:100 dilution for 30 min. After a final wash in TBS the sections were reacted

with 0.005% H_2O_2 in 3.3 diaminobenzidine tetra-hydrochloride (0.5 $mgml^{-1}$) in Tris-HCl buffer pH 7.6 until a brown colour was apparent. Sections were lightly counterstained in Mayer's haematoxylin and mounted in DPX mountant (BDH Chemicals).

Tissues were studied initially in this way but subsequently further sections were "trypsinised" after blocking with H_2O_2 in methanol, by placing in 0.1% trypsin diluted in pre-warmed 0.1% calcium chloride for varying times up to 60 min at 37° C followed by thorough washing in running water.

In control sections primary antibody was either absent or replaced with non-immune serum at dilutions from 1:25 to 1:100.

Antibodies to human plasminogen were also removed from antiserum by affinity chromatography. Ten milligrams of human plasminogen (Sigma) was dissolved in 2 ml of sodium bicarbonate buffer (0.1M pH 8.3) containing 0.5 M NaCl, which was then dialysed against two changes of the same buffer (100 ml/mg protein) to prevent unwanted coupling of Tris buffer (present in lyophilised plasminogen) to the Sepharose gel. One gram of cyanogen bromide activated Sepharose 4B (Pharmacia) was swollen for 15 min. in a solution of 1mM HCl (200ml/g) and washed in the same solution on a sintered glass filter (porosity G3). The plasminogen solution was then mixed end over end with the swollen Sepharose gel at room temperature for two hours. Residual active groups remaining

on the gel after coupling were blocked by transferring the gel to 0.2M glycine (pH 8.0) for 2h at room temperature. Excess adsorbed protein was then removed by further washing of the gel on a sintered glass filter in 5 successive changes of sodium bicarbonate buffer (0.1M, pH 8.3) containing 0.5M NaCl, followed by 0.1M acetate buffer (pH 4.0) containing 0.5M NaCl. The plasminogen-Sepharose conjugate was poured into a 10 cm glass column with an internal diameter of 6 mm (Pharmacia) attached to a peristatic pump (Pharmacia). The plasminogen antiserum (Dakopatts) was dissolved at a 1:5 dilution in 5 ml of phosphate buffered saline and loaded onto the column. The sample was eluted in phosphate buffered saline with a flow rate of 0.5ml/min. and 18 x 1 ml fractions collected. The absorbance of these fractions was measured at 280nm in a spectrophotometer to determine the presence of antibody depleted antiserum. Fractions 5 - 9 containing depleted antiserum were pooled. The depleted antiserum was used in place of primary antiserum in immunostaining as a further control.

4.2.2 Results

Sections of gingiva stained with plasminogen antiserum, without a trypsin digest stage, showed pale diffuse staining in gingival epithelium, which was most intense with a 1:25 dilution of antiserum. Sections in which primary antiserum was replaced with non-immune serum also showed background staining in the epithelium particularly with lower dilutions of non-immune serum (1:25). Sections stained only weakly

with antiserum to IgG and IgA (Table 26).

Staining of gingival mucosa with all antibodies was markedly enhanced by the inclusion of a trypsin digest stage (Table 27). Trypsinisation of sections for 10 min. was optimal. Further trypsin digestion failed to improve the quality of staining and after 30 min. in trypsin solution, sections more readily floated off the slides during subsequent staining procedures. Strong specific staining of sections was observed with IgG and IgA antiserum using dilutions as low as 1:200 - 1:400 (Table 27). Both antisera showed a different distribution of staining which was typical of the normal pattern of distribution of the antigen (Fig. 89). No background staining was observed in the absence of primary antiserum or after staining with non-immune serum. Staining with antiserum to plasminogen showed specific staining of endothelial cells lining vessels with dilutions of antibody as low as 1:200. Inflammatory cells present in the gingival mucosa also stained positively with antiserum to human plasminogen (Fig. 90). The positive cells appeared to be plasma cells. No staining was ever observed in these cells in the absence of primary antibody or after staining with non-immune serum (Fig. 91). Staining of inflammatory cells was always intracellular. The morphology of endothelial cells made it difficult to determine whether plasminogen was intracellular or simply adsorbed onto the cell surface.

In gingival epithelium, intense uniform staining was

TABLE 26

IMMUNOPEROXIDASE STAINING OF FORMALIN FIXED, PARAFFIN EMBEDDED SECTIONS OF GINGIVAL MUCOSA

Primary Antibody	Dilution	Bridging Antibody	PAP	Staining Characteristics	Intensity
Plasminogen	1:25	1:30	1:100	Pale uniform staining	++
	1:50	1:30	1:100	of epithelium in tissue	+
	1:100	1:30	1:100	sections	+
	1:200	1:30	1:100		+
Normal Rabbit Serum	1:25	1:30	1:100	Weak non-specific staining	+
	1:100	1:30	1:100	particularly in epithelium	+
Tris Buffered Saline		1:30	1:100	No background staining	-
		-	1:100	observed	-
IgG	1:200	1:30	1:100	Weak specific staining	++
IgA	1:400	1:30	1:100	showing normal pattern of distribution of antigen	++

TABLE 27

IMMUNOPEROXIDASE STAINING OF ENZYME TREATED SECTIONS OF GINGIVAL MUCOSA FIXED IN FORMALIN AND PARAFFIN EMBEDDED

Primary Antibody Dilution		Bridging Antibody	PAP	Staining Characteristics	Intensity
Plasminogen	1:25	1:30	1:100	Intense staining particularly in epithelium	++++
	1:50	1:30	1:100	Strong staining in sections particularly in epithelium,	+++
	1:100	1:30	1:100	with some staining in blood vessels in connective tissue	+++
	1:200	1:30	1:100	Uniform staining in all layers of epithelium, and endothelial cells within vessels	++
Normal rabbit serum	1:25	1:30	1:100	Background staining mainly	++
	1:100	1:30	1:100	in epithelium	+
Tris Buffered Saline		1:30	1:100	Weak background staining mainly in epithelium	+/-
		-	1:100	observed in some sections	
IgG	1:200	1:30	1:100	Strong discrete staining showing typical distribution of antigens within sections	+++
IgA	1:400	1:30	1:100		+++

FIG. 89

Section of gingival mucosa stained with polyclonal antiserum to IgA (x400 mag).

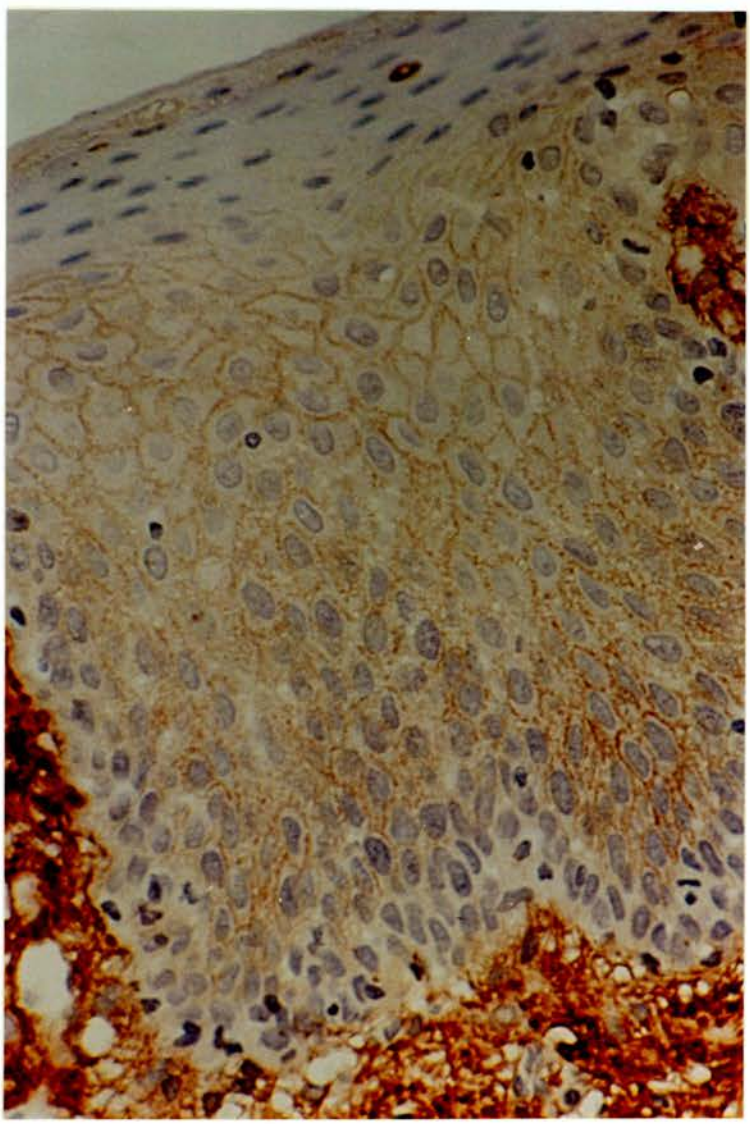
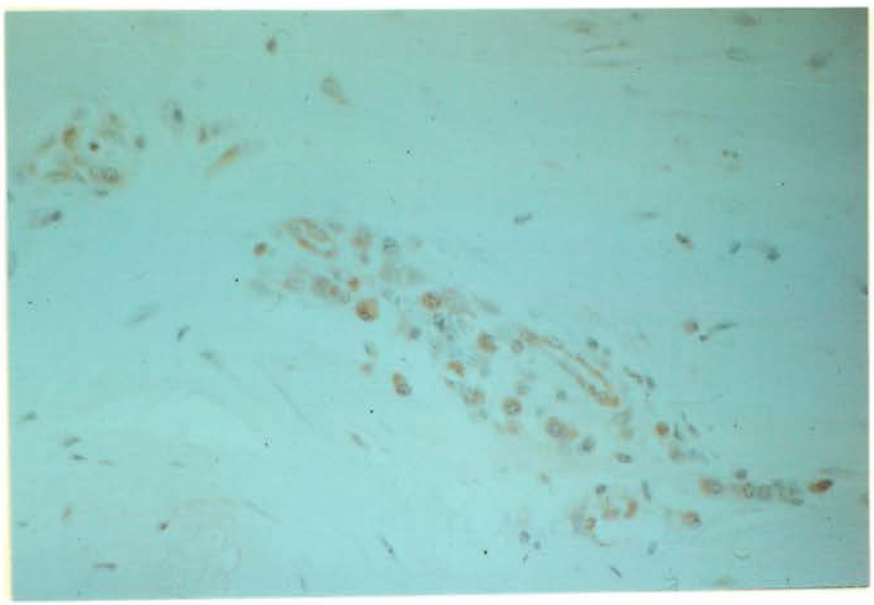


FIG. 90

Section of gingival mucosa stained with polyclonal antiserum to human plasminogen (1:200). Staining is observed in endothelial cells lining vessels and in inflammatory cells (x100 mag).

FIG. 91

Section of gingival mucosa stained with non-immune serum (1:100) (x100 mag).



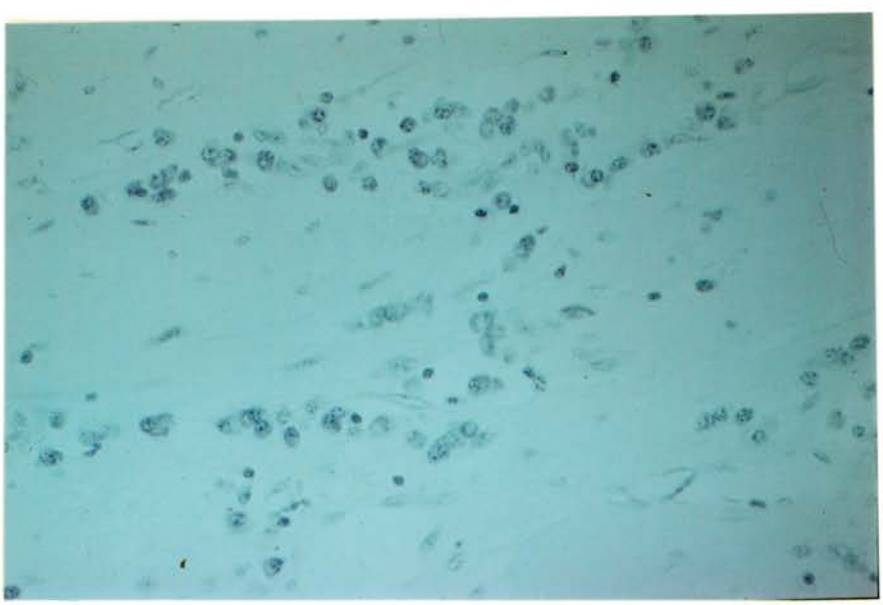
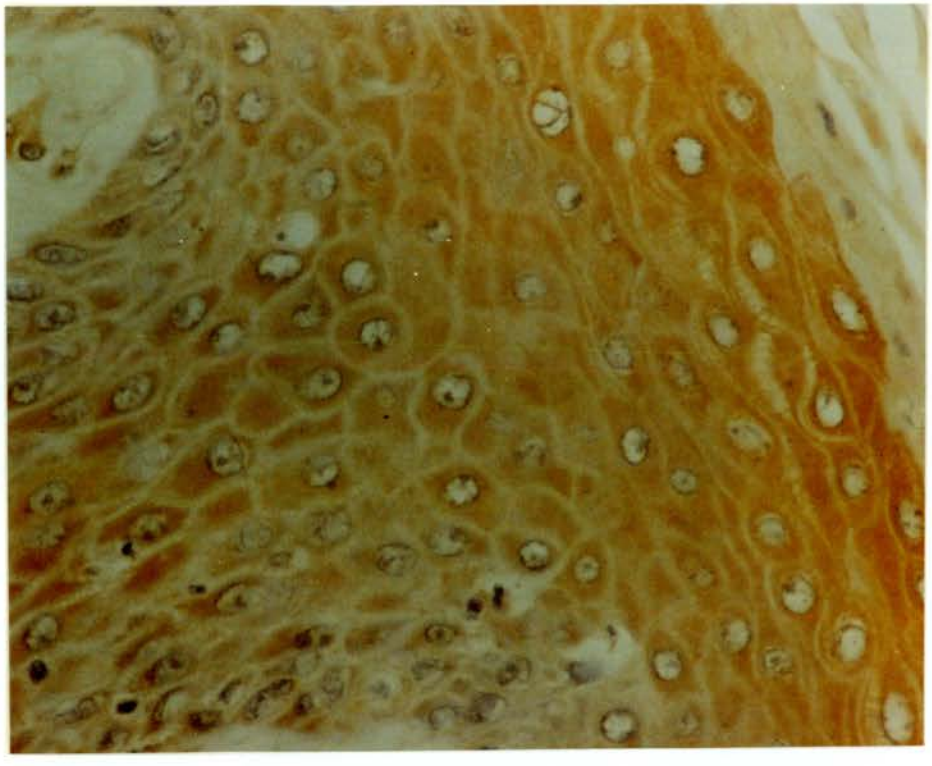


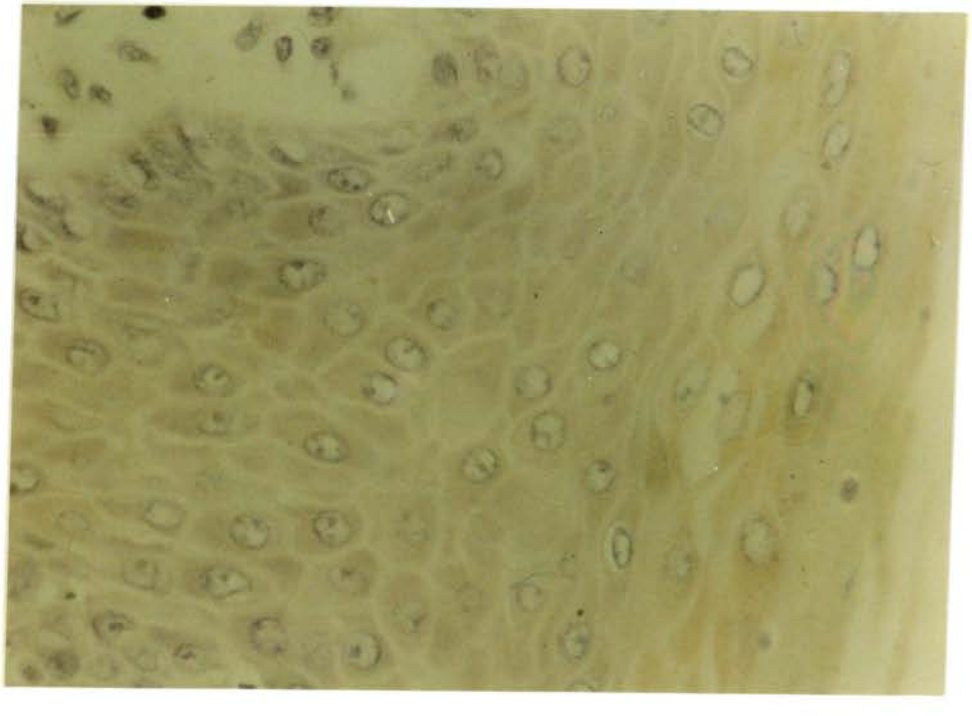
FIG. 92

Section of gingival mucosa stained with polyclonal antiserum to human plasminogen (1:200) showing uniform staining in epithelium except for surface keratin layers (x400 mag).

FIG. 93

Section of gingival mucosa stained with non-immune serum (1:100) (x400 mag).





observed in all layers of the epithelium using a 1:25 dilution of antiserum to plasminogen. Lower titres of antibody (1:100-1:200) resulted in a weaker but still uniform pattern of staining in the epithelium (Fig. 92). Indeed, weak background staining was observed in epithelium stained with non-immune serum at dilutions of 1:100 (Fig. 93) and also in occasional sections stained only with tertiary antibody.

The specificity of immunostaining with antiserum to plasminogen was further studied after depletion of antibody to human plasminogen by passing the antiserum over a plasminogen-Sepharose linked gel. Staining of endothelial cells and white blood cells was completely absent after staining with antibody depleted antiserum. Within the same series of sections, however, occasional non-specific staining was still apparent in all the epithelial layers in the absence of primary antibody or in sections stained with non-immune or antibody depleted antiserum. Immunoperoxidase staining of minor salivary glands, breast and kidney epithelium showed specific staining for plasminogen in salivary gland and mammary gland duct cells and in renal tubule cells (Figs. 94 and 95). Staining did not occur if primary antibody was omitted during the staining procedure or if antibody depleted antiserum was used.

In Summary:

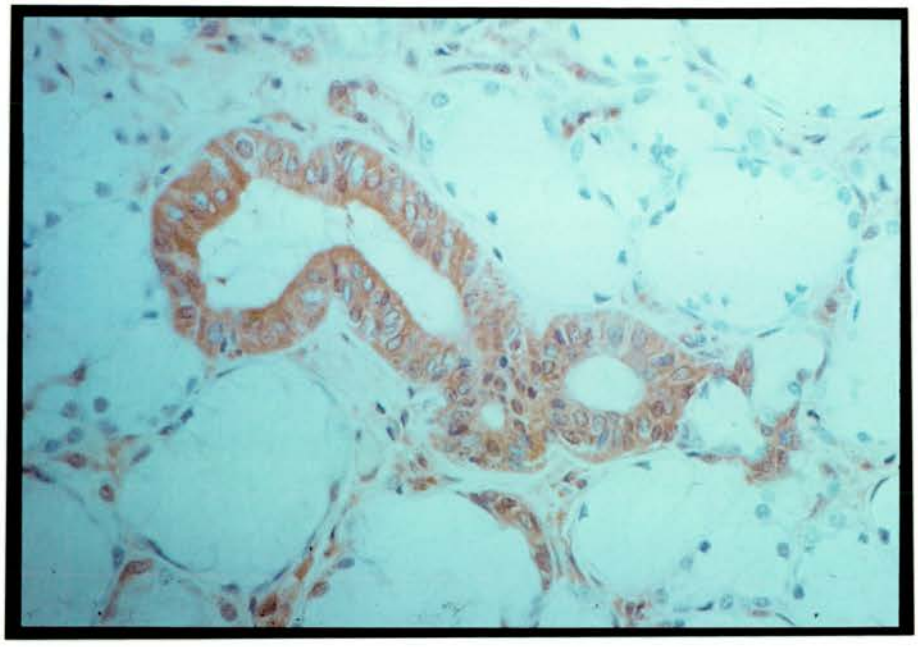
1. Immunoperoxidase staining of untrypsinised sections was weak after staining with IgG or IgA antisera and absent

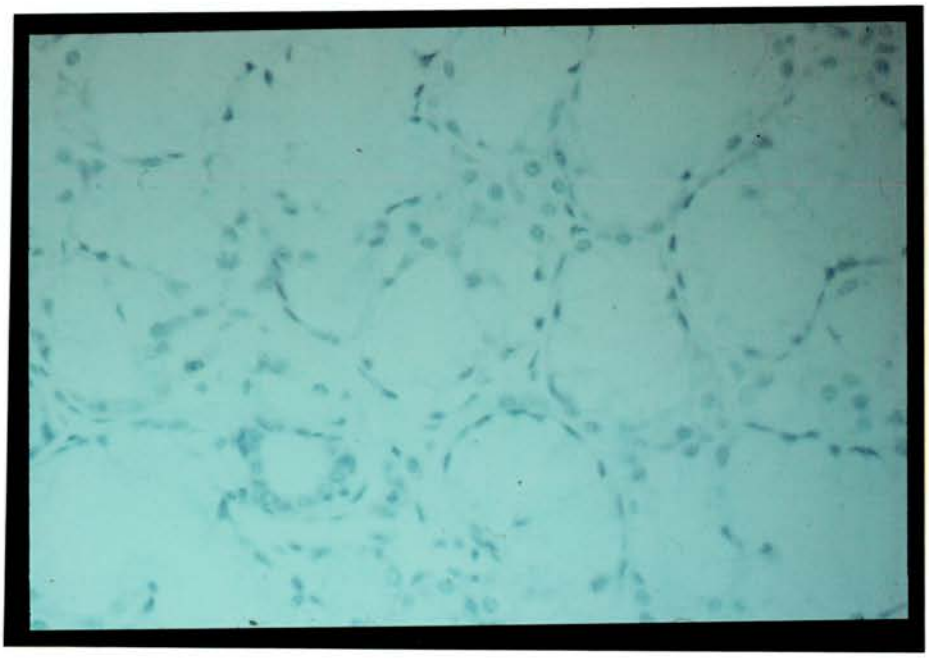
FIG. 94

Section of salivary gland stained with polyclonal antiserum to human plasminogen (1:200), showing staining in duct epithelium (x200 mag).

FIG. 95

Section of salivary gland stained with non-immune serum (1:100) (x200 mag).





after staining with low dilutions of anti-plasminogen antiserum.

2. Staining of trypsinised sections of oral mucosa with anti-plasminogen antiserum resulted in uniform staining in all layers of the gingival epithelium, however, background staining was sometimes observed in sections if primary antibody was omitted during staining procedures, after staining with antibody depleted antiserum, or with PAP complexes used alone in the staining procedure.
3. In trypsinised sections, staining with anti-plasminogen serum was specific to endothelial cells and certain inflammatory cells within the connective tissue of oral mucosa.

4.3 IMMUNOFLUORESCENCE STAINING

Immunofluorescence studies were therefore carried out to determine whether or not more specific staining for plasminogen could be obtained.

4.3.1 Materials and Methods

Sections of gingival mucosa (11) and oral sqamous cell carcinomas (13) were collected directly from surgery in serum free medium and frozen in OKT mountant in a stream of carbon dioxide vapour. Sections (4um) were cut in a cryostat and mounted on pre-cleaned slides. Sections were fixed for up to 12 hours in one of three fixatives: ice cold acetone, methanol or 70% methanol. Antiserum to human plasminogen, raised in rabbits (Dakopatts), was applied to sections at dilutions ranging from 1:10 - 1:50 in TBS for 1h at room temperature. The sections were gently washed in two changes of TBS for 15 min. and fluorescein conjugated anti-rabbit immunoglobulin (Dakopatts) applied at dilutions from 1:10 - 1:50. The sections were then washed in two changes of TBS and counterstained with Mayer's haematoxylin. Sections were mounted in buffered glycerol (BDH Chemicals). Fluorescence in sections was photographed using XPI 400 ASA film in a Leitz Dialux microscope with fluorescence filter 12 (450-490nm). In control sections primary antiserum was replaced with either PBSA, non-immune serum or antibody depleted serum.

TABLE 28

INDIRECT IMMUNOFLUORESCENCE STAINING OF FROZEN SECTIONS OF ORAL MUCOSA WITH ANTISERUM TO HUMAN PLASMINOGEN

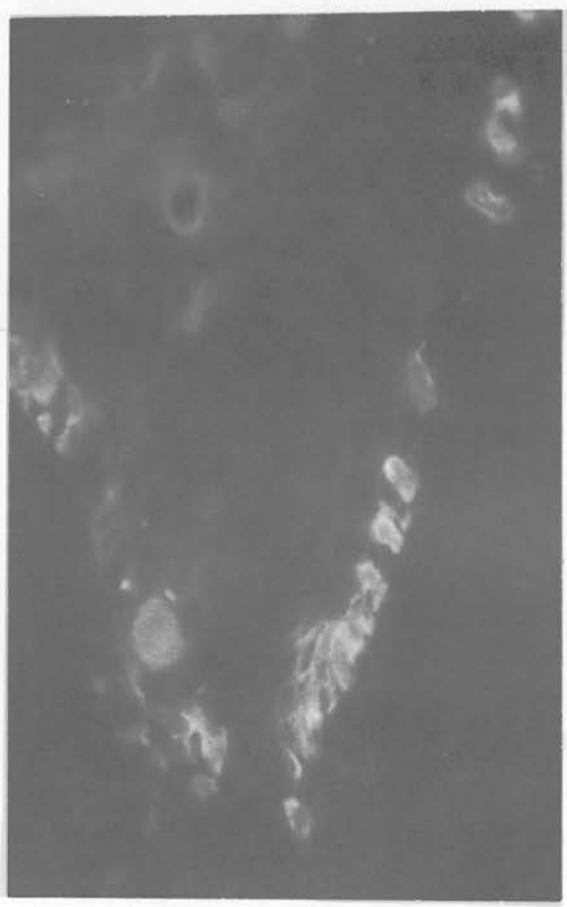
Dilution of Primary Antiserum	Dilution of FITC Conjugated anti-body	Staining Characteristics	Intensity
1:10	1:10	Strong staining in basal and prickle cell layers of epith-elium with weak background staining in other epithelial layers and connective tissue	+++
	1:20		+++
	1:50	Weak staining in basal layer of epithelium	+
1:20	1:10	Strong staining in basal and prickle cells layers of epithelium	+++
	1:20		+++
	1:50	Weak staining particularly in basal layer of epithelium	+
	1:10	Weak staining of epithelium with weak background staining of epithelium and connective tissue	+
1:50	1:20	Very weak staining in epithelium and connective tissue	+/-
	1:50	Staining weak or absent	+/-
Non Immune serum 1:20	1:20	Weak diffuse background staining in connective tissue	+/-
TBS	1:20	Staining absent	-

FIG 96

Section of gingival mucosa stained with polyclonal antiserum to human plasminogen (1:20). Fluorescence is confined mainly to basal cells (x200 mag).

FIG. 97

Light micrograph of Fig. 96 (x200 mag).



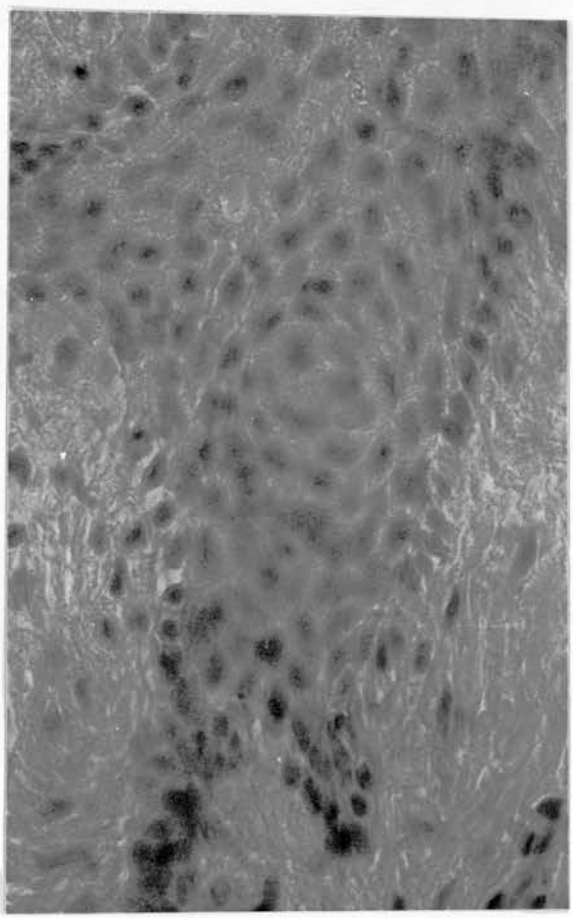
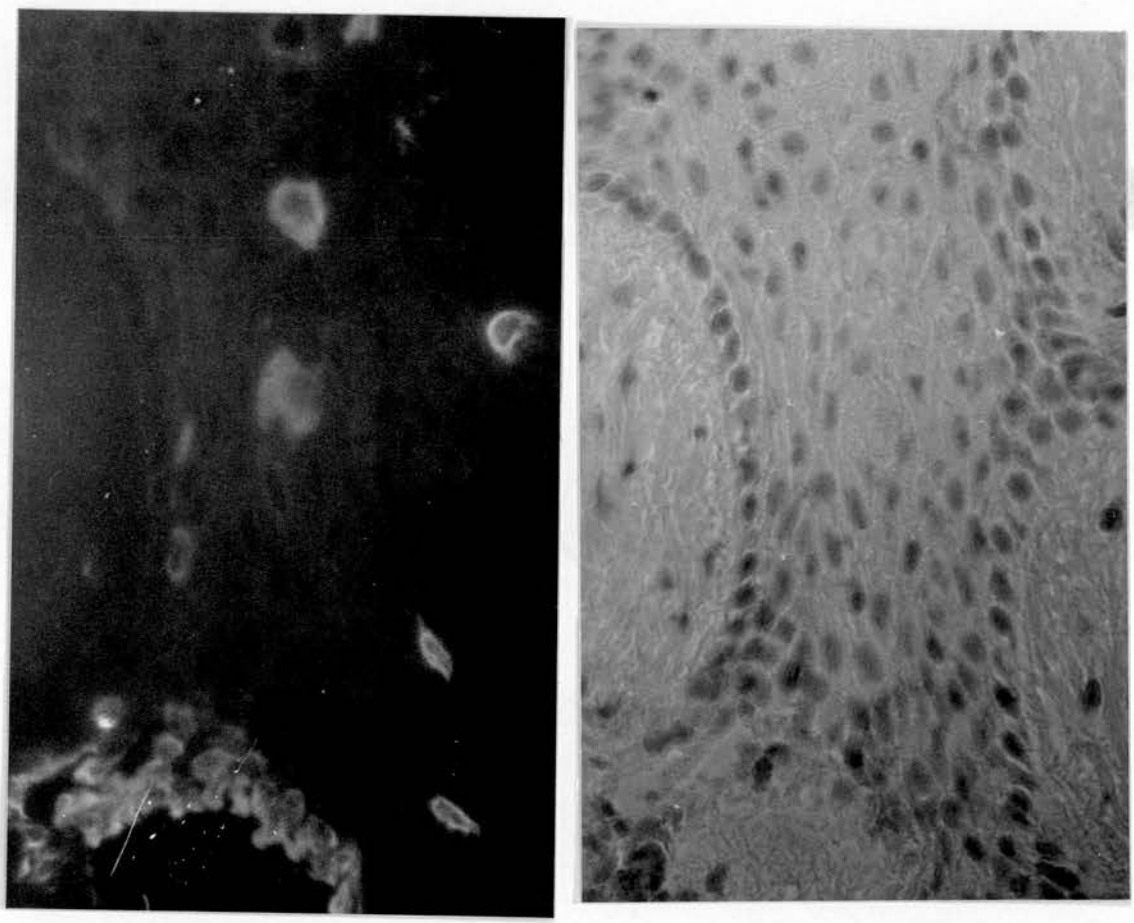


FIG. 98

Section of gingival mucoa stained with polyclonal antiserum to human plasminogen (1:20), showing distinct fluorescence in prickle cells (x200 mag).

FIG. 99

Light microscopy of Fig. 98 (x200 mag).



4.3.2 Results

No significant differences were observed in morphology or preservation of antigenicity of the sections after fixation with any of the fixatives as long as fixation times were kept short, ie less than 1h. Prolonged fixation, for example overnight, markedly reduced tissue immunoreactivity.

Optimal staining was achieved using a 1:20 dilution of plasminogen antiserum and a 1:20 dilution of FITC conjugated antiserum. Lower dilutions, particularly of primary antiserum (1:10), increased the level of background staining (Table 28). All sections of gingival mucosa stained positively for plasminogen in the basal layer of the epithelium (Figs. 96 and 97). Staining, which was intracellular, was most intense and consistent in the basal layer of epithelium, although not all basal cells stained positively in each section. Staining also occurred, although less frequently in prickle cells (Figs. 98 and 99). Endothelial cells and cells and plasma within vessels also fluoresced when stained with antiserum to plasminogen. Staining was never observed at these sites if primary antiserum was omitted during the staining procedure or if antibody depleted antiserum to plasminogen or non immune serum was used in place of the primary antiserum.

Specific staining with plasminogen antiserum was observed in eight of the thirteen oral squamous cell carcinoma specimens stained. Staining occurred mainly in tumour cells and vessel endothelium with occasional staining

in connective tissue. Examples of these are shown in Figs. 100 to 104.

No staining was observed if primary antiserum was replaced with TBS during the staining procedures or after staining with non-immune serum or antibody depleted antiserum.

Fewer of the carcinomas fluoresced after staining with antiserum to plasminogen when compared to gingival mucosa, and staining tended to be weaker.

In Summary:

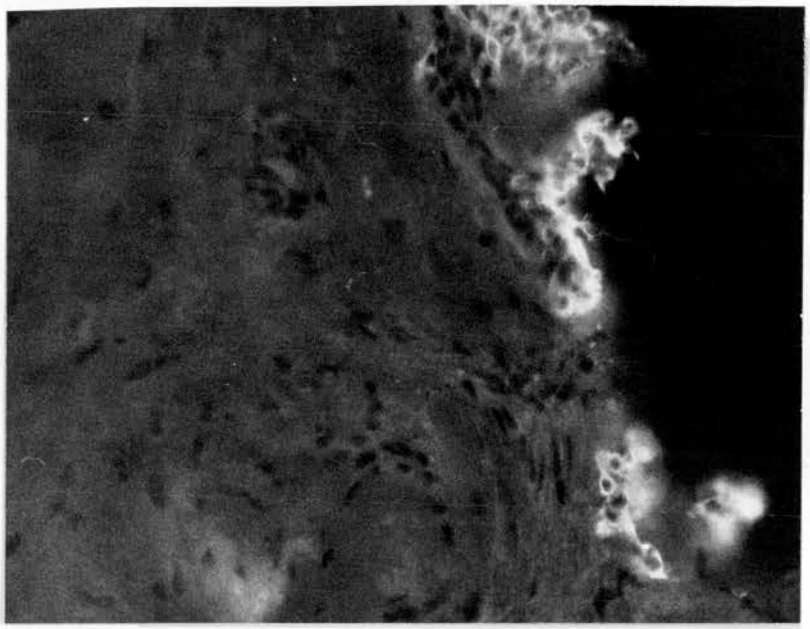
1. Highly specific staining of basal and prickle cells was observed in sections of normal oral mucosa after staining with antiserum to human plasminogen.
2. Only eight of the thirteen specimens of oral squamous cell carcinomas showed epithelial cells staining positively for plasminogen and staining tended to be weaker.
3. No staining was observed in any of these sections if primary antiserum was omitted during the staining procedure, or after staining with antibody depleted serum or non-immune serum.

FIG. 100

Section of oral squamous cell carcinoma stained with polyclonal antiserum to human plasminogen (1:20). Fluorescence is observed in fragments of epithelium on the surface of sections (x100 mag).

FIG. 101

Light micrograph of Fig. 100 (x100 mag).



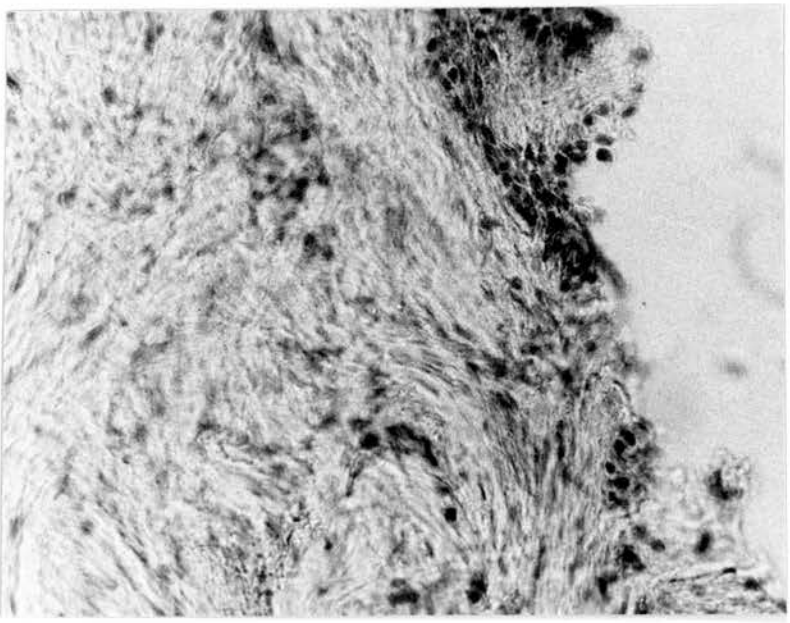
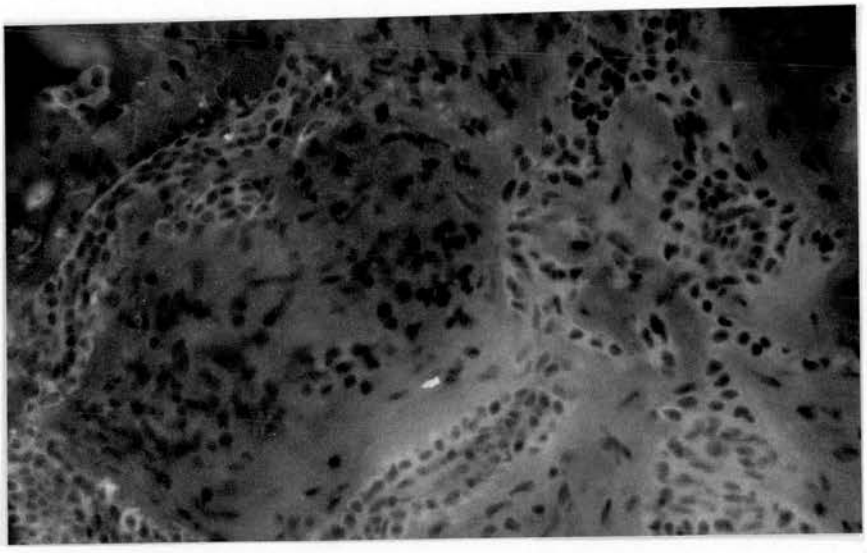


FIG. 102

Section of oral squamous cell carcinoma stained with polyclonal antiserum to human plasminogen (1:20). Fluorescence is observed in narrow cords of infiltrating tumour cells (x100 mag).

FIG. 103

Light micrograph of Fig. 102 (x100 mag).



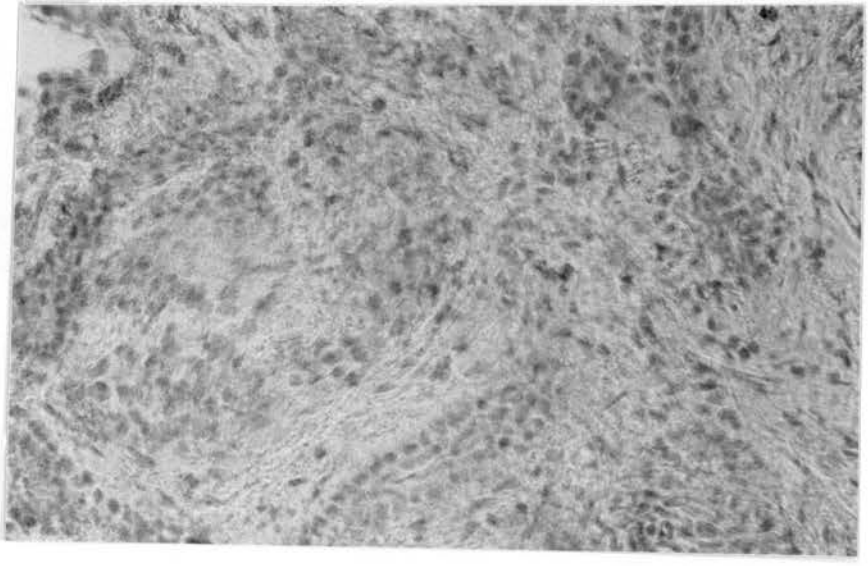
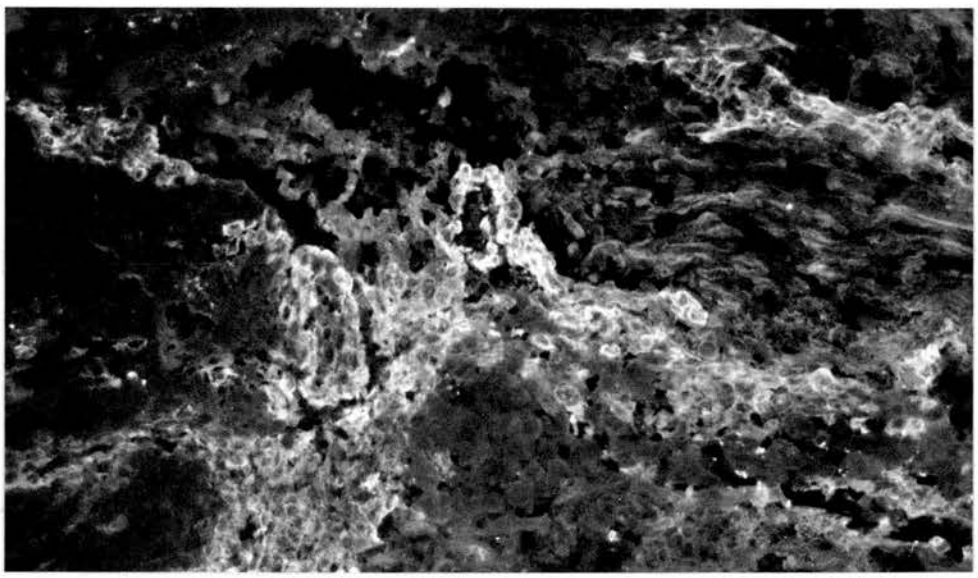


FIG. 104

Section of oral squamous cell carcinoma stained with polyclonal antiserum to human plasminogen (1:20). Bright fluorescence is observed in epithelial cells within the tumour (x200 mag).



4.4 DISCUSSION

Immunoperoxidase staining of sections of gingival mucosa untreated with proteolytic enzymes was found, in agreement with other authors, to be weak or absent. (Curran and Jones, 1978; Mepham et al 1979; Preatorius-Clausen, 1988). In earlier studies, paraffin embedded tissues fixed in unbuffered formalin, or in acetic acid containing fixatives, which increased permeability of cell membranes were shown to retain greater tissue reactivity (Curran and Jones, 1977). These authors, therefore, postulated that many antigens were not necessarily destroyed by cross linking fixatives but were made inaccessible to the large molecules used in immunoperoxidase staining. Digestion of sections, particularly by trypsin, was found to be very useful in unmasking antigens in tissues fixed in buffered formalin (Mepham et al 1979; Curran and Gregory, 1980). The process of trypsin digestion may act to increase tissue permeability or act directly on the antigen to free cross linked molecules.

Trypsin digestion of sections of gingival mucosa resulted in highly specific staining of tissues with antiserum to IgG and IgA, with no background staining in the absence of primary antiserum or after staining with non-immune serum. Similar specific staining with anti-plasminogen serum was observed in endothelial cells lining vessels and within inflammatory cells in the connective tissue of gingival mucosa sections. It is unclear, due to the morphology of endothelial cells, whether staining for

plasminogen was intracellular. Endothelial cells have been shown to readily adsorb plasminogen onto their cell surface (Hajjar et al 1986) and it seems unlikely that endothelial cells would synthesize plasminogen as it is abundant in the local environment. In contrast, plasminogen in inflammatory cells was intracytoplasmic and is possibly synthesized in these cells. The inflammatory cells were of an undetermined lineage but, on the basis of their morphology, they may have been plasma cells or of myeloid origin. Plasminogen may be associated with a phagocytic function as activated macrophages are known to secrete plasminogen activator (Unkeless et al 1974) although the presence of plasminogen within these cells has not been reported.

In the present study immunoperoxidase staining for plasminogen in gingival epithelium was less clear. In epithelium lining salivary gland ducts, breast duct and kidney tubules, staining with anti-plasminogen serum was specific. No background staining was apparent in the epithelium in sections stained in the absence of primary antibody, with non-immune serum or antibody depleted antiserum. Strong staining also occurred in stratified gingival epithelium, but staining was always uniform throughout the epithelial layers except occasionally in the surface keratin layers. These findings would suggest that plasminogen is distributed evenly in cells in all epithelial layers at any given time. In view of the complex nature of protease cascades this seems unlikely and it may be that non-specific staining occurred with the plasminogen antibody.

Indeed, weak diffuse staining was also seen in these sections in the absence of primary antibody or in sections stained with non-immune or antibody depleted antiserum. Background staining was also occasionally observed in sections stained only with the PAP complex. Cross reactivity of other antibodies in the primary antiserum may account for the lack of specificity of staining, however, it does not account for the staining in sections in the absence of this antiserum. Non specific binding of antibodies to cytokeratin tonofilaments has been reported by other authors (Curran and Gregory, 1980). It may be that this is the cause of non specific staining in sections in the present study. Antigenicity of plasminogen may also have been affected by other stages in the paraffin embedding process. Matthews (1981) demonstrated that reagents other than cross linking fixatives have a markedly deleterious effect on tissue reactivity. In view of the difficulties encountered using this technique, no other stages in the process were investigated in this study.

Immunofluorescence staining of frozen sections of gingival mucosa, fixed very briefly in either methanol or cold acetone, showed more limited staining with anti-plasminogen serum. Fluorescing cells were confined mainly to the basal layer of epithelium although not all cells in the basal layer stained positively. Occasional cells in the prickle cell layer also stained positively with antiserum to plasminogen. Isseroff and Rifkin (1983) and Nakagawa et al

(1984) similarly detected plasminogen in the basal layer of epithelium in skin using indirect immunofluorescence, however, unlike the findings in the present study, staining was confined only to the basal layer of the epithelium.

Staining in gingival epithelium was intracytoplasmic but it is uncertain whether or not plasminogen is synthesized in these cells. Basement membranes do not exclude plasma proteins (Squier and Rooney, 1976) and plasma proteins have been demonstrated, by immunoperoxidase staining, in gingival epithelium (Genco et al 1974; Brandtzaeg, 1974; Coruh and Mason, 1980). In most of these studies IgG and IgA were demonstrated in intercellular spaces in the epithelium. In a later study, however, Reibel (1982) showed that larger molecules such as IgM and fibrinogen (M_r 340,000) were present both inter and intracellularly. Intracellular fibrinogen was, however, confined to damaged cells in inflammed oral mucosa. Plasminogen (M_r 92,000) is a smaller molecule than fibrinogen and may also diffuse across the basement membrane. Plasminogen is already known to bind to basement membrane components and to a number of cell types including endothelial cells, and to a limited extent fibroblasts and smooth muscle cells (Hajjar et al 1986; Knusden et al 1986) although it is unclear whether plasminogen actually enters cells.

The presence of plasminogen in gingival epithelium can therefore be explained, either by diffusion into the epithelium, or by its synthesis in these cells. If positive staining for plasminogen results from diffusion of this

plasma protein across the basement membrane, the presence of both a diffusion gradient and intercellular staining may be expected, but neither were observed in sections stained with immunoperoxidase or immunofluorescence staining techniques.

Plasminogen may be synthesised by basal cells which retain this plasminogen as they move into the prickle cell layer. This may explain the less frequent staining for plasminogen in these cells. The absence of staining for plasminogen elsewhere requires further investigation.

A possible role for plasminogen in the differentiation of epithelium has been suggested by _in vitro_ studies. Green (1977) collected squames from supernatants of cultured foreskin epithelium and found after further incubation of the sloughed cells, that they required the presence of plasminogen containing serum for nuclear digestion to occur. Since the nuclear envelope develops before nuclear digestion is complete, the envelope must either remain permeable to plasminogen or plasminogen must be present before the nuclear envelope has formed.

No plasminogen was detected in the cornified layers of epithelium in gingival mucosa. Plasminogen has similarly been detected by others only in the basal layer of epidermis (Isseroff and Rifkin, 1983; Nakagawa _et al_ 1984). These findings are inconsistent with a role for plasminogen in terminal differentiation _in vivo_, although it is possible that sufficient plasminogen is present in cells for nuclear digestion to occur but not in sufficient quantity to be

detected by immunofluorescence staining.

A role for plasminogen in tumour epithelial cells is less difficult to suggest. Malignant cells express the common property of invasion and destruction of surrounding tissues. The process of cell invasion has generally been considered to involve the production of proteases (Laug et al 1975; Jones and deClerk, 1980; Kramer, 1982) and plasmin has been shown to initiate the breakdown of the extracellular matrix components fibronectin and laminin (Liotta et al 1981). Whether plasminogen is bound to tumour cells or is synthesized in situ, an enhanced generation of plasmin possibly by tumour epithelial plasminogen activator, could be an important event in invasion and early implantation of micro-metastases in blood vessel walls or stroma.

In one study, plasminogen has been demonstrated by immunofluorescence staining to bind to the surface of tumour cells in colon carcinoma (Burtin et al 1985). Plasminogen stained the contours of tumour glandular structures, foci of tumour cells and isolated tumour cells but no intracytoplasmic staining was observed. The authors suggested that plasminogen exuding from blood capillaries within the tumour diffuses into the stroma and binds to tumour cells. Plasmin once formed there may then play a role in invasion of tumour cells, dissociation of tumour cells from the main mass and the production of necrosis within the tumour mass.

In this study, plasminogen was detected in eight of the thirteen specimens of oral squamous cell carcinoma stained by

immunofluorescence. In five of the positive biopsy specimens tissue had been excised from the junction between normal and malignant tissue, so should be representative of the tumour invasion zone. The precise location from which tumour tissue was taken in other specimens was not known. Plasminogen staining in oral squamous cell carcinomas was intracellular and not simply bound to the cell surface. Again the question of intracellular synthesis versus endocytosis of plasminogen within these cells cannot be answered using these techniques, although it is interesting to note that Burtin et al (1985) failed to detect intracytoplasmic staining in similar cells.

Using in vitro studies, Bogenmann and Jones (1983) have demonstrated that degradation of matrix proteins by the tumour cell lines HT1080 (human fibrosarcoma), B161F (mouse melanoma) and a human rhabdomyosarcoma was plasminogen dependent particularly in low cell density cultures. The dependency for plasminogen changed with cell density and time in culture, degradation becoming largely plasminogen independent. Further breakdown of matrix proteins, at high cell densities, may be achieved by other serine proteases or by cathepsins which are known to be synthesised by some melanoma cell lines (Sloane et al 1982). Such modulation of plasminogen dependent degradation of matrix proteins suggests that the pattern of hydrolytic enzymes produced in vivo may be responsive to environmental influences such as tumour site or tumour cell density. Biswas et al (1978) found that the

implantation site of V-2 carcinoma in rabbits determined whether or not extractable collagenase was produced. In another study Cajot et al (1986) characterised and quantitated plasminogen activator expressed by human primary colon carcinomas and colon carcinoma cell lines Col 112 and Col 115 inoculated into outbred mice. They found that subcutaneous grafts of Col 112 and Col 115 behaved as non-invasive well circumscribed tumours, while those cells injected into the gastro-intestinal tract had a highly invasive growth pattern. Their studies also demonstrated a good correlation between the production of plasminogen activator and the malignant phenotype. Gastro-intestinal extracts contained higher PA-PAI complexes than subcutaneous implants, therefore lower plasminogen activator production was not simply under inhibitor control. Alterations in plasminogen activator activity may well result from modulatory effects of the host tissue environment. Such effects may include the availability of plasminogen at tumour sites.

Of particular importance to tumour invasion was the finding that plasminogen, which is bound to a surface, is activated with much greater efficiency than fluid phase plasminogen (Hajjar et al 1986: Knudsen et al 1986). In addition, plasminogen which binds to matrix proteins, possibly via lysine binding sites, is protected from the fast acting inhibitor alpha-2-antiplasmin. This inhibitor relies for its action on binding to plasminogen and plasmin via lysine binding sites (Harpel, 1981), however, when these are

occupied by matrix proteins even a 100 fold excess of inhibitor was not able to abolish plasmin activity in experimental systems (Knudsen et al 1986). Only small amounts of plasminogen and plasminogen activator may therefore be required for lytic activity to occur and protection from inhibitors would also result in prolongation of plasmin activity. The synthesis of plasminogen by oral squamous cell carcinomas, which may also synthesise plasminogen activators would, therefore, confer an obvious advantage for tumour invasion.

4.5 SUMMARY

Plasminogen has been detected by indirect immunofluorescence in the basal and prickle cells in gingival epithelium. Its function in these cells remains unclear but it may be involved in aspects of terminal differentiation and cell movement in epithelium.

Plasminogen was also detected intracellularly in tumour epithelial cells. Its presence in these cells, particularly at the site of tumour invasion, may enhance local generation of plasmin and invasion of tumour cells into the surrounding tissue.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

The association between malignancy and increased plasminogen activator activity was observed as early as 1925 by Fischer. This experimental approach involved assaying for plasminogen activators in fragments of normal and neoplastic tissue and has disadvantages. Such samples include not only tumour elements but vascular endothelium, blood and inflammatory cells all of which may produce plasminogen activator.

Interest in the role of plasminogen activator in the modulation of tumour behaviour was renewed in the 1970's when increased synthesis of plasminogen activator was detected after transformation of cells *in vitro* (Ossowski *et al* 1973; Unkeless *et al* 1974; Pollack *et al* 1974; Quigley *et al* 1974).

Since this time a large number of studies have sought to correlate enhanced plasminogen activator synthesis with various parameters; including morphological transformation of cells (Balduzzi and Murphy, 1975; Decker *et al* 1977), tumourigenicity (Pollack *et al* 1975; Laug *et al* 1975; Adelman *et al* 1981; Ramshaw *et al* 1986) and metastatic capacity of transformed cells lines or neoplastic tissues (Wang *et al* 1980; Eisenbach *et al* 1985). Whilst there appeared, in many cases, to be an obvious correlation between these events, apparently conflicting findings were reported

(Mott et al 1974; Wolf and Goldberg, 1976; Nicholson et al 1976; Montesano et al 1977; Whur et al 1980) in which plasminogen activator was either not necessary or was insufficient for tumour invasion.

The emergence of uPA as the key enzyme in malignant tissues (Markus et al 1983; Mira-y-Lopez et al 1983; Ossowski et al 1983; Burtin et al 1985; Khoga et al 1985; Camiolo and Greco, 1986; Mignatti et al 1986; Busso et al 1987) and the discovery of pro-enzyme forms of plasminogen activators (Kielberg et al 1985) and specific inhibitors of both uPA and tPA (Sprengers and Kluft, 1987) have meant that much of the work to date requires re-evaluation.

Further more, it was recognised that for plasminogen activator to have a causal role in tumour invasion, the overall activator content may not necessarily be higher than that of normal tissues but that the activator must at least be located in areas where tumour invasion occurs. Analysis, therefore, of the occurrence of activators in tumour tissues should be coupled with histochemical studies of plasminogen activators.

Early histochemical studies were performed using the fibrin overlay technique (Peterson and Zettergen, 1970; Peterson et al. 1975) but those failed to distinguish between uPA and tPA activity. Since fibrin potentiates the action of tPA it is possible that mainly tissue activator was localised in these studies. Many of these studies also failed to localise plasminogen activators in cancer cells (Weiss and Beller, 1969; Svanberg et al 1975; Newstead et al 1976;

Ljungner et al 1984) which may be the result of PA-inhibitors in these tissues.

The development of specific antibodies to uPA and tPA and the use of non-fibrin based assays has allowed a clearer picture of the role of plasminogen activator in neoplasia to emerge. To date, however, few have been reported. In human colon carcinomas, uPA has been identified by immunocytochemistry in neoplastic cells and is particularly intense in transitional areas between normal and neoplastic tissue and in areas of tumour invasion (Markus et al 1983; Burtin et al 1985; Khoga et al 1985).

In considering the role of plasminogen activators in cancer several questions must now be addressed. Investigations should elucidate whether transformed cells or specific tumours contain plasminogen activators, how this property influences the functional behaviour of neoplastic cells and whether elimination of these plasminogen activators inhibits tumour invasion and metastases.

In the present study it has been demonstrated that normal oral epithelium contains plasminogen activators, although their precise functions in epithelium are still ill understood. Epithelial cells in oral squamous cell carcinomas also contain plasminogen activators of both uPA and tPA types although staining for activators was weak and may be due to the presence of inhibitors in the tissue. However, plasminogen, which may protect plasminogen activators from the action of inhibitors, was also identified

in sections of oral squamous cell carcinomas at the junction between normal and malignant tissues.

Whether sufficient enzyme is present in these tissues to modulate tumour behaviour is unknown. Regulation of plasmin activity occurs at many levels:

At a subcellular level, there may be increased transcription of PA genes, repression of constitutive biosynthesis of PA-inhibitors or increased synthesis of enzymes which activate pro-PA. Indeed, it has recently been shown in HeLa cells *in vitro* that increased transcription of PA genes occurs in response to tumour promoters (Waller and Schleuning, 1985). Activation of pro-enzymes of plasminogen activator represent a further point at which the plasmin cascade may be controlled. The assumption that pro-enzymes will be activated by trace amounts of proteases in the extracellular compartment is incorrect. Evidence has shown that a proportion of plasminogen activator secreted from cells can remain in the single chain form (Skriver *et al* 1984). The mechanisms which control pro-enzyme activation may involve PA-inhibitors; however, as yet, little information concerning these mechanisms has been reported. In addition, inhibitors of proteases which are abundant in tissues, may further modulate extracellular proteolytic activity. Furthermore, plasminogen activator production in cells *in vitro* is subject to hormonal control at physiological concentrations of these hormones. These may exert qualitatively similar effects on tumours *in vivo*.

In experimental studies, which seek to define a role

for plasminogen activator in neoplasia, plasminogen activators must be identified, appropriate methods of assaying enzyme activity must be employed and the relative contribution of pro-enzymes and their inhibitors assayed.

For many human diseases, tissue culture is often the only method with which to study disease processes. In the present study an *in vitro* model was developed in which to quantitate plasminogen activators in normal and neoplastic epithelial cultures. Many early efforts to grow epithelium *in vitro* have been hampered by the rapid differentiation of epithelium and its overgrowth by fibroblasts. Advances in tissue culture techniques particularly the emergence of new media for keratinocyte growth (Tsao *et al* 1982; Boyce and Ham, 1983) have altered the balance in favour of cellular proliferation and have extended the lifespan of such cultures.

In this study the size of biopsy material limited methods available for culturing tissue and oral keratinocytes were grown in explant culture in a minimally supplemented medium. Oral epithelium *in vitro* forms a multilayered stratifying tissue similar to parent tissue *in vivo*. The pattern of growth and differentiation of epithelium *in vitro* is remarkably alike in a number of tissue culture systems (Holbrook and Hennings, 1983) and the reproducibility of this model makes it useful in the study of normal physiology and disease processes.

Cultures established from oral squamous cell

carcinomas were shown, by EM and immunoperoxidase staining, to contain nearly pure populations of epithelial cells. These cells retained a morphology associated with the malignant phenotype, a reduced capacity to differentiate, altered cell-cell and cell-substrate adhesion, increased cell migration and increased growth rate. Tumour heterogeneity does, however, constitutes a major problem in the investigation of cancer cells. Different areas of the tumour, and particularly sub-populations selected *in vitro*, may differ with respect to the properties being investigated. By using primary cultures, the genetic and phenoytpic properties of the cultures were as close as possible to the parent tissue (Fidler and Hart, 1978).

Using a fibrin based assay, plasminogen activator activity in tumour cultures grown for four weeks *in vitro* was shown to be significantly lower than that of gingival keratinocyte cultures. The predominant activator synthesized by both normal and malignant tissues was uPA and differences in enzyme activity were not, therefore, the results of the potentiating action of fibrin on tPA activity in the samples.

SDS-PAGE separates plasminogen activators from non-complexed and complexed inhibitors in samples. In comparing normal and tumour extracts expressing equivalent levels of activity on fibrin plates, tumours were found to contain much higher levels of plasminogen activator activity than gingival keratinocyte cultures. Tumour cultures therefore contain both higher levels of plasminogen activator and PA-inhibitors than their equivalent normal counterpart. Tumour

supernatants were also shown to contain more PA-PAI complexes than gingival keratinocyte cultures. These observations, therefore, support the hypothesis that malignant cells synthesize and secrete more plasminogen activator than normal tissues.

Findings in this work indicate that plasminogen activators, particularly uPA, and its substrate, plasminogen, are present in normal epithelium and neoplastic tissue both *in vivo* and *in vitro*.

Whether these activators function in tumour invasion and metastases is unknown. The hormonal milieu of the local environment is obviously an important factor. In addition, increased synthesis of PA-inhibitor by tumours may ablate proteolytic activity at the site of invasion and immunocytochemical localisation of these complexes *in vivo* may help clarify their role. Interestingly, plasminogen, which was also detected in transitional areas between normal and malignant tissue in oral squamous cell carcinomas, protects plasminogen activator from the action of plasmin inhibitors in *in vitro* systems (Knudsen *et al* 1986). No evidence is available concerning the interactions between plasminogen and PA-inhibitors.

Through the use of animal models of tumour invasion, involving the injection of cultured cells, a causative role for plasminogen activator in tissue degradation and metastases can be further investigated.

Inhibition of tumour formation and metastases in animals by

specific inhibitors of uPA and tPA, and hormonal modulation of enzyme activity would provide sound evidence concerning the role of plasminogen activators in tumour biology.

Few studies of this nature have been reported in the literature. Recently, however, Mira-y-Lopez et al (1983) found that hydrocortisone reversibly blocked the growth of mammary tumours in mice and that growth rate of the tumours resumed at control rates if exogenously supplied hydrocortisone was withdrawn. They also found that pre-treatment with hydrocortisone could delay the appearance of primary tumours and reduced their numbers. Hydrocortisone reduction of tumours in mice was correlated with a reduction in plasminogen activator activity in tumour cells. Later Busso et al (1987) showed that the reduction in plasminogen activator activity of dexamethasone treated cells resulted from a decrease in the concentration of uPA mRNA and a decrease in the synthesis of single chain pro-enzyme but also from increased synthesis of PA-inhibitor. The hormonal milieu of the local environment is therefore of considerable importance. Certain polypeptides for example are also known to increase plasminogen activator activity (Lee and Weinstein, 1978; Dayer et al 1981, Sudol et al 1985). Since many tumour cells are capable of synthesizing their own growth hormones by autocrine secretion (DeLarco and Todaro, 1978) this mechanism may play an important role in the aggressiveness of a particular tumour. Furthermore, Laiho et al (1986) have shown that transforming growth factor-beta is capable of inducing plasminogen activator synthesis, in

fibroblasts. Enhancement of secretion of plasminogen activator in adjacent normal tissues by tumour cell TGF-beta may lead to an increase in plasminogen activator activity in the vicinity of tumour cells. The site of injection of tumour xenografts into nude mice has also been shown to influence the production of plasminogen activator synthesis by these tumours (Cajot et al 1986). Enzyme synthesis by these tumours was correlated with tumour invasiveness. Such differences in plasminogen activator production may well be the result of differences in expression of hormones at these sites.

Conclusive evidence of the involvement of plasminogen activator synthesis and metastases has been shown in only one study to date. Ossowski and Reich (1983) found that antibodies which specifically inhibited the enzyme activity of human uPA but not chicken uPA decreased metastases of human tumour (Hep-3) to chicken lung after transplantation onto the chorioallantoic membrane of chick embryos. Since growth at the site of injection was not inhibited, then uPA must play an important role at an early stage in the metastatic process.

Further such studies as these are obviously required to gain a better understanding of the role of plasminogen activators in tumour invasion. The in vitro model described in the present study in which plasminogen activators and their inhibitors have been identified and characterised should provide a useful tool to assist the further

elucidation of these processes.

APPENDIX - TRADE INDEX

Agar Scientific,
66A Cambridge Road,
Stanstead,
Essex.

BDH Chemical Ltd.,
Broom Road,
Poole,
Dorset.
BH12 4NN

Behringwerke,
Hoechst UK Ltd.,
50 Salisbury Road,
Hounslow,
Middlesex.
TW4 6JH.

Biopool, AB.,
Box 1454,
S901 24 UMEA,
Sweden.

Corning Ltd.,
Laboratory Division,
Stone,
Staffs.
ST15 OBG

Dako Ltd.,
22 The Arcade,
The Octagon,
High Wycombe,
Bucks.
HP11 2HT

Flow Laboratories,
PO Box 17,
Second Avenue Industrial Estate,
Irvine,
Ayrshire.
KA12 8NB

Ilford Ltd.,
Distribution Centre,
PO Box 21,
Mobberly,
Knutsford,
Cheshire.
WA16 7HA

Kabi Diagnostics,
Kabivitrum House,
Riverside Way,
Uxbridge,
Middlesex.

Kodak Ltd.,
PO Box 66,
Station Road,
Hemel Hempstead,
Herts.
HP1 1JU

Leo Laboratories,
Longwick Road,
Princes Risborough,
Aylesbury,
Bucks.
HP17 9RR

May and Baker Ltd.,
Liverpool Road,
Barton Moss,
Manchester.
M30 7RT

Miles Scientific,
Naperville,
Illnois,
USA 60540

Ortho Diagnostics Systems Ltd.,
Enterprise House,
Station Road,
Loudwater,
High Wycombe,
Bucks.
HP10 9UF

Parke Davis and Co.,
Mitchell House,
Southampton Road,
Eastleigh,
Hants.
SO5 5RY

Sigma Chemical Co. Ltd.,
Fancy Road,
Poole,
Dorset.
BH17 7NH

Sterilin Ltd.,
Sterilin House,
Clockhouse Lane,
Feltham,
Middlesex.
TW14 8QS

TAAB Laboratories Equipment Ltd.,
40 Grovelands Road,
Reading,
Berks.

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